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NORMAL LIMITS OF URINARY COPROPORPHYRIN EXCRETION DETERMINED BY AN IMPROVED METHOD

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IN A recent communication[1] an improved procedure for measuring urinary coproporphyrin was presented and contrasted with a previously employed "long" method. The average normal per diem (twenty-four-hour) excretion observed with the "long" method was 54 μg per day.[2] The average value obtained with the new method on a closely corresponding sample of normal subjects was approximately 160 μg per day. This threefold increase in the average twenty-four-hour urine value obtained with the new method was shown to be due to (a) maintenance of a neutral or alkaline urine during the period of collection, (b) conversion and measurement of coproporphyrin precursor, (c) more efficient extraction, and (d) more efficient fluorimetry.

The following report will present data defining the normal limits of twenty-four-hour urinary coproporphyrin excretion as determined with this improved procedure. The effect of variations in customary alcohol consumption by normal individuals will be presented simultaneously.

METHODS

Analytical Procedure.—Details of the improved procedure which utilizes a 5 ml. aliquot from a twenty-four-hour urine sample have been reported.[1] Studies since then have shown that normal urine contains several other porphyrins which are partially soluble in the ethyl acetate-acetic acid mixture employed in the analytical procedure. However only a very small fraction of the porphyrin quantitated is not coproporphyrin.[3]

Case Material.—The cases studied consisted originally of 746 presumably healthy men called in for examination during a veterans' follow-up survey. However, thirteen were eliminated because of evidence of abnormality such as abnormal liver biopsies or significant exposure to noxious agents. Thirteen additional cases were normal in all respects but consumed excessive amounts of alcohol (the equivalent in alcohol content of one pint or more of whisky each day). They were studied separately. This left 720 cases to comprise the norm group. The average age of this group was 30 ± 5 years, average weight 162 ± 24 pounds, and average surface area 1.86 ± 0.14 square meters. Approximately half of the group had had viral hepatitis five years previously; the others were their controls. None of the

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subjects had any evidence of liver disease, by criteria reported on elsewhere,[4] after exhaustive study with a battery of liver function tests. The coproporphyrin frequency distributions for the two subgroups corresponded closely with respect to their means and standard deviations. They were therefore combined to form the norm group. During the course of the collections, thirty-five of the urine specimens were inadequate or unavailable for analysis for various extraneous reasons, so the final distribution studies are based on 698 subjects.

A second sample of 52 normal men, consisting largely of medical students, was observed at the University Hospital. In addition, analyses were obtained on specimens collected by 51 young women, mostly nursing students.

Alcohol Consumption.—Ratings of alcohol intake were available for the sample of veterans comprising the norm group. The rating scheme[4] involved a ten point scale designed to provide a semiquantitative measure of approximate alcohol intake irrespective of beverage consumed. A rating of "one" represented a customary intake approximately equivalent to a fifth of whisky each day; of "two," one pint each day; of "three," one-half pint each day. A rating of "nine" represented alcohol consumption equivalent to "an occasional drink per month or a number of drinks on rare occasions." A rating of "ten" indicated no alcohol intake. Intermediate ratings represented stepwise decrements in alcohol consumption, the higher the rating the less the intake.

Seasonal Effect.—No primary attempt was made to study the effect of seasonal variations on coproporphyrin excretion. However, certain incidental findings were noted which are reported here as preliminary observations. Specimens of thirty of the fifty-one normal women were studied in September and October. The remaining twenty-one cases were observed the following April and May. A significant average difference was observed. Therefore the larger group of normal men was reviewed. There were 200 specimens collected during the fall months (October, November, December), and 172 collected during the spring (April, May, June). These formed two groups which were contrasted with respect to average coproporphyrin excretion. The fifty-two medical students were studied during the month of May.

Analysis of the Data.—Frequency distributions were plotted for the various alcohol rating (AR) groups. Means ($\bar{x}$) and standard deviations (S) were recorded, and where appropriate, tests of significance of differences (critical ratios) between means were calculated. Where frequency distributions were based on sufficiently large groups, the coproporphyrin values which were exceeded by 5 per cent and 1 per cent of the cases were recorded.

RESULTS

Coproporphyrin Frequency Distributions.—The subjects were divided into six subgroupings according to alcohol rating (AR). Frequency distributions of coproporphyrin excretion for these groupings are recorded in Fig. 1 along with their means and standard deviations. The highest average coproporphyrin excretion was 232 μg per day obtained on the group with the highest alcohol consumption. The lowest average value was 166 μg per day obtained on the group that did not drink alcoholic beverages. The low value is comparable to that of 163 ± 53 μg per day (range 65 to 279) obtained on fifty-two additional normal men with presumably minimal alcohol consumption, though an exact rating for these cases was not obtained. Between the extremes, there was a sequential decrease in average coproporphyrin excretion as the consumption of alcohol decreased. It is apparent that proper evaluation of any observed coproporphyrin value requires a knowledge of ordinary alcohol consumption no matter how slight.

All of the individuals comprising the norm group were gainfully employed men who were pronounced in good health after careful physical examination.

All had essentially normal liver function test results which were comparatively uninfluenced by variations in alcohol consumption.[4] The group as a whole may be considered representative of normal men of that age range in a general sense. Thirteen individuals had alcohol ratings of "one" or "two." This represented consumption of the equivalent of at least a pint of whisky per day, an amount sufficient to interfere with nutrition eventually. These cases were therefore omitted when the various AR groups were combined to form a single

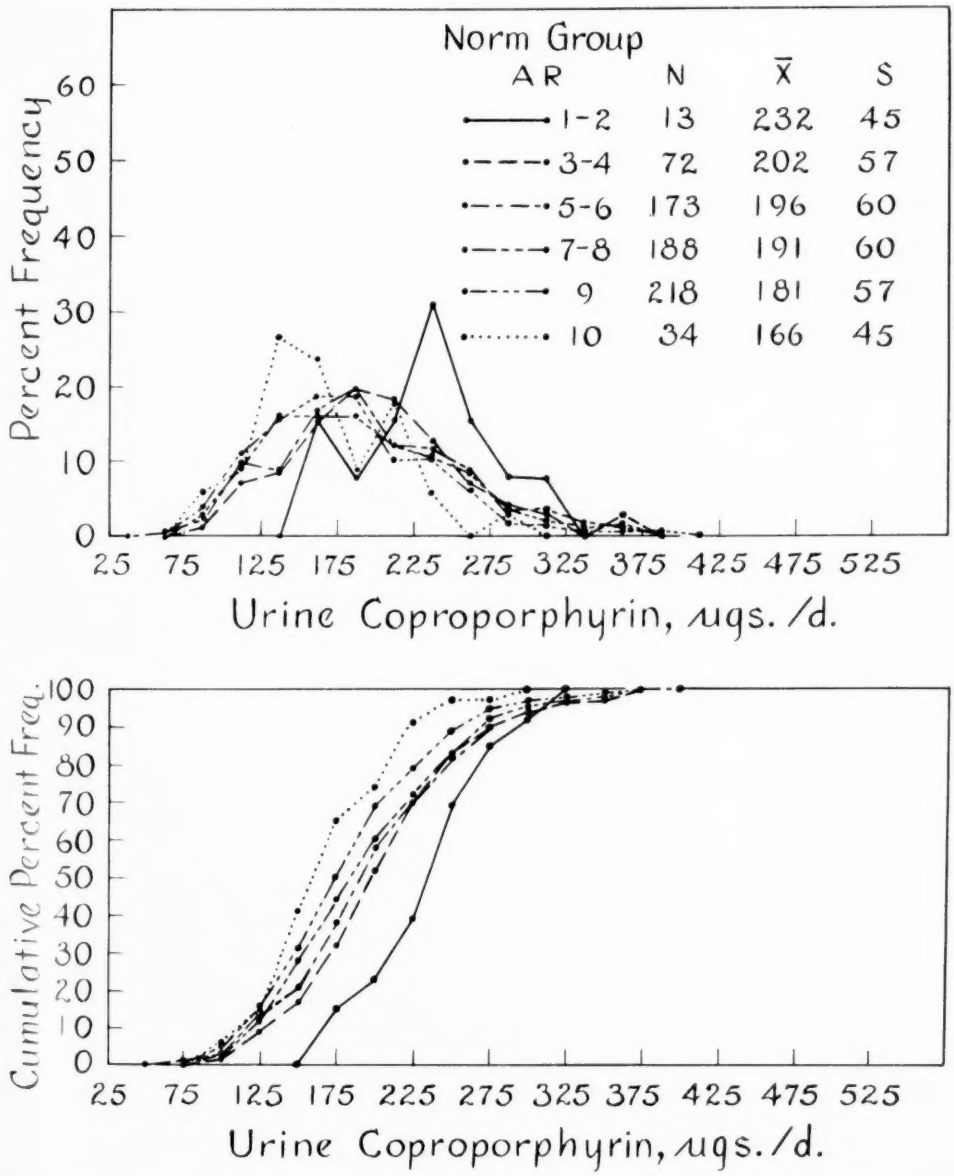

Fig. 1.—Frequency distributions of coproporphyrin excretion, by alcohol consumption groups.

norm group. The frequency distribution for this combined group is plotted in Fig. 2. The mean coproporphyrin excretion when alcohol consumption is thus disregarded is 189 ± 59 μg per day. Of these 685 cases, 4.5 per cent had values of 300 μg or greater. The average coproporphyrin excretion for 579 cases with intermediate ratings, AR 5-9, was identical; viz, 189 ± 59 μg per day.

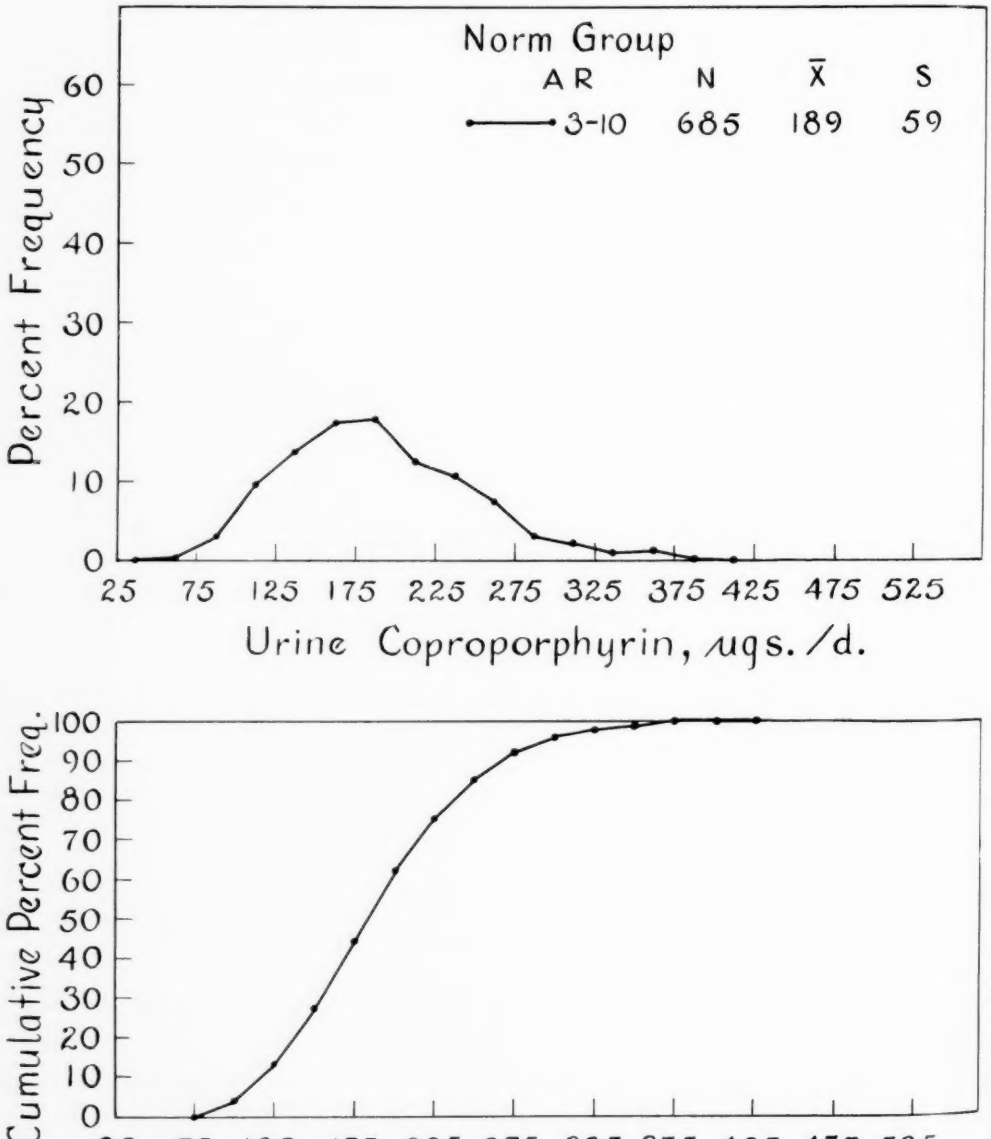

Fig. 2.—Frequency distributions of coproporphyrin excretion for norm group as a whole.

Limits of Normal.—On the basis of these distributions, limits may be set for coproporphyrin excretion beyond which a normal individual would be unlikely to fall. For various levels of alcohol consumption we have recorded in Table I the coproporphyrin values which were exceeded in an increasing or decreasing direction by 5 per cent and 1 per cent of the cases. From these tables it is evident that one may take 300 μg per day as a satisfactory upper limit of normal and 100 μg per day as a satisfactory lower limit of normal in most instances. A more appropriate upper limit for those cases with more than moderate alcohol consumption would be 325 to 350 μg per day; and for those with very little or no intake, 275 μg per day.

Sex Differences.—The average per diem coproporphyrin excretion for the group of fifty-one normal women was 134 ± 42 μg per day. The range of values was 48 to 268 μg per day. Two-thirds of these individuals did not drink any alcoholic beverage. Exact ratings on the remainder were unavailable. The mean for this group of women is significantly lower than the value of 166 ± 45 μg per day obtained on the thirty-four men with AR 10 (critical ratio = 3.28). A tentative upper limit of normal for women may be set at 275 μg per day.

TABLE I. COPROPORPHYRIN VALUES ABOVE WHICH AND BELOW WHICH 5 PER CENT AND 1 PER CENT OF NORMAL MEN FALL, BY ALCOHOL GROUPING

ALCOHOL RATING	NUMBER OF CASES	OBSERVED LIMITS			
		UPPER		LOWER	
		5%	1%	5%	1%
5-6	173	310	354	104	78
7-8	188	299	359	107	82
9	218	275	361	100	77
5-9	579	297	358	103	79
3-10	685	296	360	103	80

Seasonal Variation.—The mean coproporphyrin excretion for the thirty normal women observed during September and October was 147 ± 45 μg per day. The range of values was 68 to 268 μg per day. This is to be contrasted with the value of 114 ± 30 μg per day obtained on the twenty-one women observed during the following months of April and May. The range of values for this group was 48 to 155 μg per day. The mean difference of 33 μg per day is statistically significant (critical ratio = 3.16).

Review of the 200 men who were observed during the fall months revealed an average coproporphyrin excretion of 205 ± 68 μg per day. This was significantly greater than the mean of 179 ± 60 μg per day obtained on 172 men observed during the spring. There is thus suggestive evidence that coproporphyrin excretion is greater in fall than in spring.

DISCUSSION

The normal values observed with the improved method are considerably higher than the highest (120 μg per day) previously reported.[5] The increase was at least threefold over other methods reported. It seems likely that heretofore most investigators have failed to recover the largest fraction of the coproporphyrin excreted, probably primarily because the urine pH was not

controlled and the portion present as precursor was lost. Preliminary data suggest that this threefold increase is not observed in patients with abnormally high excretion of coproporphyrin. Presumably in these instances proportionately less coproporphyrin was lost with the "long" method. Further studies of this phenomenon are in progress.

Observers have previously noted increased coproporphyrin excretion shortly following ingestion of alcoholic beverages. Franke and Fikentscher[6] and Franke[7] originally observed increases following relatively small amounts of alcoholic beverages. Sutherland and Watson[8] observed marked rises in coproporphyrin excretion in chronic alcoholics during and following the consumption of large amounts of alcohol. The results reported herein indicate that this effect of alcohol is evident even in the normal individual who has not recently ingested alcoholic beverages. In fact, comparatively small amounts of alcohol ingested intermittently produce a demonstrable average increase in coproporphyrin excretion. Also, relatively slight increments in alcohol consumption result in demonstrable increments in average coproporphyrin excretion.

The sex difference in coproporphyrin excretion is of interest, though by itself the exact significance of the observation is not clear. Of importance in this respect are the studies of Hoffbauer and co-workers[9] who found lower urinary coproporphyrin excretion in female than in male rats. Administration of androgens to the females after castration resulted in significant elevation of the coproporphyrin values. The combination of castration and estrogen administration to the males resulted in significant reduction of coproporphyrin values. The lower values in the women of the present study are correlated with their lower hemoglobin concentrations. This difference is to be contrasted with the converse observation of greater erythrocyte protoporphyrin, erythrocyte coproporphyrin, and reticulocyte values in women of a corresponding age.[10]

No relationship was noted between body weight and coproporphyrin excretion, based upon single determinations, for the large sample of men. The correlation coefficient was –0.12, insignificantly different from zero. This absence of relationship is not necessarily in conflict with the observation of Strait and associates,[11] based on five cases, that "the *mean* amount of coproporphyrin excreted per day may be expressed as a linear function of the weight of the adult individual." It suggests, however, that variability within individuals is greater than variability due to weight differences.

The incidental finding of seasonal variation in coproporphyrin excretion was evident in both sexes. The fall-spring differential appeared to be greater for women. In the light of these observations one might anticipate seasonal variations in hemoglobin concentration on careful study.

SUMMARY

The distribution of normal values for urinary coproporphyrin excretion determined by an improved method was studied in 698 normal men and 51 normal women.

The mean coproporphyrin excretion for men, disregarding alcohol consumption, was 189 ± 59 μg per day. There was a sequential increase in average

coproporphyrin excretion as customary alcohol intake increased. Slight increments in intake resulted in distinct increases in coproporphyrin excretion.

The lowest average value obtained in males who consumed no alcoholic beverages was 166 μg per day. The highest average value obtained in those with heavy daily alcohol consumption was 232 μg per day.

On the basis of the frequency distributions observed an upper limit of normal was set at 300 μg per day for most cases, and a lower limit of normal at 100 μg per day. For those individuals with moderately heavy alcohol consumption the upper limit should be raised to 350 μg per day, and for those abstaining completely the limit should be lowered to 275 μg per day.

The average coproporphyrin excretion for women (134 $\pm$ 42 μg per day) was significantly less than for men. A tentative upper limit of normal for women was set at 275 μg per day.

For both sexes the average coproporphyrin excretion during fall was significantly greater than that during spring.

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RECOVERY OF AUREOMYCIN FROM THE GASTROINTESTINAL TRACT FOLLOWING INTRAVENOUS ADMINISTRATION*

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INFORMATION concerning the secretion into the gastrointestinal tract of intravenously administered Aureomycin is of clinical and fundamental interest. Aureomycin is sometimes indicated in the treatment of gastrointestinal disease when the patient is unable to take oral medication. Also, intravenous Aureomycin has been thought to be effective in the treatment of acute hepatic insufficiency and hepatic coma.[1, 2] It is of interest to know whether significant levels of the antibiotic are attained in the intestinal tract in such instances, since the beneficial action might thus be secondary to the effect of Aureomycin on the bacterial flora, and a postulation of effect in the liver itself would be unnecessary.

Herrell and Heilman[3] demonstrated active concentration of Aureomycin in the bile of patients with T tubes. They found the level in the bile to be six to eight times that in the serum. Zaslow, Hewlett, and Lorry[4] studied the concentration of Aureomycin in the gall bladder contents of 25 patients given the antibiotic before cholecystectomy, and showed significant levels when the cystic duct was not obstructed. Zaslow, Hewlett, and Lorry[5] measured Aureomycin excretion in bile drained by T tube in eight patients immediately after cholecystectomy, and noted levels as high at one hour after injection as at three. Two patients had transient absence of Aureomycin in the bile; one of these was jaundiced and the other had evidence of mild parenchymal liver damage. Kraus and associates[6] observed secretion of Aureomycin into the saliva in low concentration. Jacob and associates[7] found changes in the fecal flora of man and dogs during administration of Aureomycin intravenously.

The purpose of the present investigation was to assay the contents of several portions of the gastrointestinal tract for Aureomycin content after intravenous administration of the drug. Concentrations were determined in the gastric secretion, the contents of the third portion of the duodenum, and in material obtained by lavage of the colon.

MATERIALS AND METHODS

A total of 19 patients was studied, and all had normal renal and cardiac function by routine tests. Four patients with presumedly normal stomachs were tested for secretion into the gastric juice. A Levine tube was inserted with the patient in a fasting state, and the stomach aspirated as completely as possible. Five hundred milligrams of Aureo-

From the Department of Medicine, University of Minnesota School of Medicine.

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*The Aureomycin used was furnished through the courtesy of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

**Present address: Peter Bent Brigham Hospital, Boston, Mass.

mycin were then injected intravenously over a 5 to 10 minute interval. Specimens were aspirated at various times thereafter and the stomach was emptied as completely as possible at each collection.

Assay of Aureomycin excretion into the third portion of the duodenum was performed with 9 patients. A Camus tube was passed to the ligament of Treitz under fluoroscopic control. The Camus tube is a flexible, rigid-walled tube with a stilette which permits rapid intubation of the duodenum, and has been described by Alessandri and associates.[8] Ten milliliters of olive oil were injected into the tube initially to promote emptying of the gall bladder, and a control specimen was aspirated fifteen minutes later. Then 500 mg. of Aureomycin was injected intravenously, accompanied in three instances by 350 mg. of bromsulfalein. Samples were aspirated at intervals except in two instances where continuous suction was used. Of the nine patients studied, one was normal and the others had disease of the liver or biliary tract.

Five patients were examined for excretion of Aureomycin into the colon. None had symptoms referable to the intestinal tract, and all had normal barium enemas. Breakfast was withheld and a tapwater enema was given before the procedure started. An enema tube was then inserted to a distance of 40 centimeters. Thirty milliliters of 2 per cent sodium chloride solution was injected through the tubing, retained fifteen minutes, then evacuated and saved as a control specimen. The patient then received 500 mg. of Aureomycin intravenously, and one and one-half to two hours later the colon lavage procedure was repeated. Only a few tiny specks of feces ever occurred in these retention enemas.

The retention enema procedure described above was also performed on the distal segment of the colon in one patient with a double barrel transverse colostomy. This patient had had a spontaneous perforation of the sigmoid colon two months previously, requiring the colostomy. At the time of the experiment the distal portion was normal by barium enema study and by sigmoidoscopy. When the colostomy was repaired a short time later, no pathology was noted except a localized area of scarring in the wall of the sigmoid colon.

Assay Method.—The method used for determination of Aureomycin concentration was a modification of the diffusion technique with paper discs, and was suggested by Johansson.* Bacillus number 5, a strain of *Bacillus cereus var. mycoides*, known to be very sensitive to Aureomycin[9] was the test organism. It was grown on nutrient agar slants for four days at room temperature and removed from each tube by washing with 3 ml. of distilled water. The washings were pooled and heat shocked at 80° C. for eleven minutes. Assay culture medium consisted of yeast extract 2 Gm., glucose 1 Gm., tryptone 5 Gm., agar 15 Gm., and distilled water 1,000 ml., buffered at pH 7. The medium was heated until liquid, cooled to 50° C., and mixed with 0.2 ml. of *Bacillus* number 5 spore suspension per 100 ml. of medium. Pour plates were made using 10 ml. for each standard flat-bottomed Petri dish.

The material to be assayed was either tested immediately or frozen with dry ice and kept at 0° C. In the latter case, assay was always done within two weeks of collection. The material was centrifuged at 4,000 to 5,000 r.p.m. for fifteen minutes and the supernatant removed. Filter paper discs were grasped with fine forceps and dipped into the supernatant as recommended by DeBeer and Sherwood.[10] The discs were analytical filter paper ½ inch in diameter. A standard series of Aureomycin concentrations was prepared for each assay as follows: 0.5 μg, 1 μg, 2.5 μg, 5 μg, and 10 μg per milliliter.

Plates were incubated at room temperature for eighteen hours, by which time a rather thick growth of colonies was visible. The discs showing Aureomycin activity were surrounded by a clear circular area of complete inhibition extending through the depth of the medium. A control specimen was always obtained for assay before Aureomycin was given. If this control gave nonspecific inhibition, the diameter of inhibition was subtracted from that of the test discs. The assay was considered invalid if the control

*Dr. K. R. Johansson, Department of Bacteriology and Immunology, University of Minnesota.

gave as much inhibition as the 0.5 μg per milliliter standard solution. A graph was made from the standard Aureomycin solutions, plotting concentration against diameter of zone of inhibition, and activity of unknowns was obtained from the curve.

RESULTS

Assay Method.—The diffusion method of Aureomycin assay gave reproducible results, and duplicate determinations showed but slight differences or were identical. The standard curves were similar to those described elsewhere for this method.[12] The diffusion technique presented several advantages for assay of contaminated material. Little time was required in manipulation which was an important consideration in working with Aureomycin. If the supernatant was not completely sterile after centrifuging, the assay was not invalidated. Two or three contaminant colonies within the zone of inhibition could be recognized by gross morphology as different from *Bacillus cereus*, and therefore could be disregarded. Incubation at room temperature also served to inhibit contaminants. The sensitivity of the method was adequate for the purpose of this experiment. Table I shows the zones of inhibition obtained in a typical standard curve.

TABLE I. ASSAY OF AUREOMYCIN STANDARDS

CONCENTRATION OF AUREOMYCIN IN μG PER ML.	DIAMETER OF ZONE OF INHIBITION IN CM.
0.5	0.6
1.0	0.9
2.5	1.4
5	1.8
10	2.2

Assay of Gastric Contents.—Aureomycin in relatively low concentration was found in the gastric contents of all four patients tested. Concentration appeared to be as high at one hour as at two, and the levels seemed too high to be explained on the basis of contamination with swallowed saliva. None of the control specimens produced nonspecific inhibition of *Bacillus* No. 5. The specimens of gastric contents were not tested for bilirubin, but their color never suggested the presence of bile. Table II demonstrates the Aureomycin levels obtained.

TABLE II. AUREOMYCIN LEVELS IN GASTRIC CONTENTS (μG PER MILLILITER)

PATIENT	TIME AFTER INJECTION					OCCULT BLOOD
	15 MIN.	30 MIN.	45 MIN.	60 MIN.	120 MIN.	
A. M.	0.7	2.2	2.2	2.4		0
A. B.				2.5	1.7	0
C. M.				1.0	1.0	0
M. H.				1.2	1.3	0

Assay of Duodenal Contents.—The results of these determinations are shown in Table III. The duodenal contents reflected the high concentration of Aureomycin noted by others to be present in the bile. Patients A. C. and H. P., who had jaundice due to incomplete stricture of the common bile duct,

TABLE III. AUREOMYCIN LEVELS IN DUODENAL CONTENTS

PATIENT	DIAGNOSIS	TSB*	BSP†	CCF‡	AUREOMYCIN CONCENTRATION IN μG PER MILLILITER TIME IN HOURS										SPECIMENS TESTED FOR	
					½	1	2	3	4	5	6	7	8	11	BLOOD	BILE
T. T.	Normal liver	0.5	0%	0		2.5	4.1	3.3		5.4				2.6	trace	present
A. C.	Postoperative stricture of common bile duct	4.6	–	0	1.0	6.5	5.0	6.5							0	present
H. P.	Postoperative stricture of common bile duct	12.5	28%	1+		2.5	3.0	3.0	2.0						0	present
D. J.	Laennec's cirrhosis	12.5	27%	4+		0.5	0.5	0.9	2.5	4.6	0.9	0.9	2.5		0	present
A. K.	Infectious hepatitis	26.5	–	4+		0	1.0	0.5	0.2						0	present
I. G.	Hodgkin's infiltration of liver	2.9	42%	4+	0	0									0	present
C. T.	Carcinoma of pancreas with complete biliary obstruction	22.0	36%	0		0	0.8	0.8	0.7						0	absent

*Total serum bilirubin in mg. per cent.
†Bromsulfalein test, forty-five minute retention. (5 mg. per kilogram of dye given.)
‡Cephalincholesterol test, twenty-four hour reading.

showed prompt appearance of substantial levels of Aureomycin. Patients A. K. and D. J., with evidence of pronounced parenchymal liver damage, had some lag in the development of peak levels. Patient I. G., also with severe parenchymal liver damage, had no demonstrable Aureomycin level in the brief collection period. Patient C. T., who had complete biliary obstruction, exhibited some Aureomycin activity in the duodenal contents. This material had the gross appearance and pH of gastric juice, and a test for bilirubin was negative. All other patients had demonstrable bile in the duodenal contents. Two patients, one with hepatitis and the other with cholangiolitic cirrhosis, are not included as their control specimens gave considerable nonspecific inhibition of *Bacillus* No. 5. Control specimens in patients A. K. and C. T. showed a little nonspecific inhibition. Patients H. P., D. J., and A. K. received 350 mg. of bromsulfalein with the Aureomycin; the dye was present by qualitative test in all samples.

Assay of Colon Lavage Material.—This study was designed to demonstrate possible secretion of Aureomycin by the lower portion of the bowel. The lower 40 cm. of large intestine was lavaged with 2 per cent solution of sodium chloride. The Aureomycin levels obtained were quite high, and in patient T. D. far exceeded the highest concentration used in the standards. None of the control specimens from the colon showed nonspecific inhibition of *Bacillus* No. 5. Patient R. T. had study of the distal segment of a double barrel transverse colostomy. The colon distal to the colostomy did not have normal physiologic function, as there was difficulty in expelling the retention enema. Both the control and test enemas were retained two hours for that reason. Relatively low levels of Aureomycin occurred in the test specimens, but secretion in this instance was definitely through the colonic mucosa. Table IV demonstrates the concentrations obtained.

TABLE IV. AUREOMYCIN LEVELS IN COLON LAVAGE (μG PER ML.)

PATIENT	HOURS AFTER INJECTION				OCCULT BLOOD
	1½	2	4½	18	
M. H.	2.4				0
F. C.		4.5			0
A. M.		6.0			0
I. R.	5.0				0
T. D.		10.0			0
R. T.		0.5	0.5	0.7	trace

DISCUSSION

Aureomycin rapidly appeared in the contents of the gastrointestinal tract following intravenous injection. Levels obtained in the stomach, and in the duodenum of one patient with complete common bile duct obstruction, did not indicate active concentration of the Aureomycin in these secretions. The duodenal contents, on the other hand, exhibited Aureomycin in high concentration even when partial biliary obstruction was present. The bile thus provided the most concentrated source of Aureomycin for excretion into the

intestinal tract when the antibiotic was given intravenously. Three patients who had parenchymal liver damage showed a lag in the development of peak levels of Aureomycin in the duodenum.

The appearance of Aureomycin in the colon one and one-half to two hours after intravenous administration could be due to a very rapid descent of the drug from the duodenum to the large bowel. Against this possibility is the fact that an insignificant amount of fecal material appeared in the retention enema fluid. Also, the rather high concentrations of Aureomycin obtained are not well explained on that basis. It seems more likely that the Aureomycin was secreted along the lower portion of the intestinal tract. Added evidence for this explanation was the appearance, in one case tested, of the antibiotic in the segment of colon distal to a double barrel transverse colostomy.

On the basis of the present studies, intravenously administered Aureomycin should be effective in the lumen of the gastrointestinal tract. The mode of action of Aureomycin in hepatic insufficiency is unknown. The present results indicate that the drug quickly comes in contact with the intestinal flora of the colon, and may well exert its beneficial effects through bacterial inhibition alone.

SUMMARY

1. Aureomycin given intravenously was recovered rapidly, in significant levels, in the stomach, duodenum, and colon.

2. The Aureomycin found in the colon suggests secretion of the drug along the lower portion of the bowel.

Acknowledgment.—The author is indebted to Dr. F. W. Hoffbauer for his helpful advice in these studies.

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THE URINARY AND FECAL EXCRETION OF I^{131} FROM LABELED SODIUM-L-THYROXINE IN VARIOUS THYROID STATES AND IN EUTHYROIDS WITH "BILE FISTULAS"

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VARIOUS thyroxine preparations have been found to be less active when given orally than when given intravenously.[1] This difference has been ascribed to relatively poor absorption of thyroxine from the gut. The per cent absorption of a dose of thyroxine given orally to animals has been reported to vary from 6 per cent[2] to 50 per cent.[3] Albert, Tenney, and Larenz[4] found 20-25 per cent of I^{131}-labeled l-thyroxine was absorbed from the isolated small or large bowel of rats within five hours after injection into the lumen of these organs. Thompson and his associates[5] showed that di-sodium thyroxine is 100 times, and monosodium thyroxine 30 times, as effective as crystalline thyroxine when given by mouth for the treatment of myxedema. Recently, however, Myant and Pochin[6] gave I^{131} labeled Na-l-thyroxine to euthyroid human adults orally and by vein. They found that after oral administration of this compound, the accumulation of radioiodine, I^{131}, in the thyroid, urine, and feces, and the composition of the plasma radioiodine were comparable to the values obtained after intravenous administration of the same compound. These figures were consistent with complete absorption of Na-l-thyroxine from the gastrointestinal tract. We wish to submit additional evidence that this same preparation of thyroxine may be well absorbed but re-excreted through the gut wall into its lumen. It was of additional interest that accumulative thyroxine-derived I^{131} excretion in feces and urine was similar in various thyroid states.

METHOD AND MATERIALS

Four adults were studied. M. L. I. was a 21-year-old woman with well-differentiated carcinoma of the thyroid with metastases to cervical nodes and lungs. A "total" surgical thyroidectomy had been done July, 1950. She had been treated with I^{131} until classical myxedema had become apparent in August, 1951. She was then started on desiccated thyroid. The dosage of thyroid was increased rapidly so that she had been on 15 grains a day for two months at the time she was used in this study on Feb. 4, 1952. She had classical symptoms and signs of thyrotoxicosis at this time, with a basal metabolic rate of +29 per cent, basal pulse rate of 140 beats per minute, and plasma cholesterol of 110 mg. per cent. She was maintained on this dosage of desiccated thyroid until the completion of our study. She is referred to hereafter as having "artificial hyperthyroidism." A. K. was a euthyroid hebephrenic schizophrenic man, 45 years old, studied Mar. 8, 1952, with cholecystostomy performed on Jan. 28, 1951, for cholelithiasis. Cholangiograms on Jan. 14, 1952 and Mar. 3,

From the Department of Internal Medicine, University Hospital.

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The I^{131}-labeled sodium-l-thyroxine was purchased from Abbott Laboratories, Chicago, Ill.

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1952 showed a residual stone in the lower end of the common duct. He maintained acholic stools, normal liver function tests, and an average daily bile volume of 648 ml. per twenty-four hours with T-tube in situ during the period of study. F. O. was also a euthyroid "bile fistula" patient, a 53-year-old woman, post-cholecystostomy, but with a stone remaining in the common duct distal to the T-tube. A cholangiogram had been performed on April 7, 1952. Our study was started on April 8, 1952, sixteen days after cholecystostomy. A battery of liver function tests, including alkaline phosphatase, was within normal limits. Z. N. suffered from untreated classical myxedema secondary to thyroidectomy. He was 68 years of age. His basal metabolic rate was −43 per cent. His protein-bound iodine was 2.9 micrograms per cent, and inorganic iodine 2.2 micrograms per cent. His liver battery was also normal.

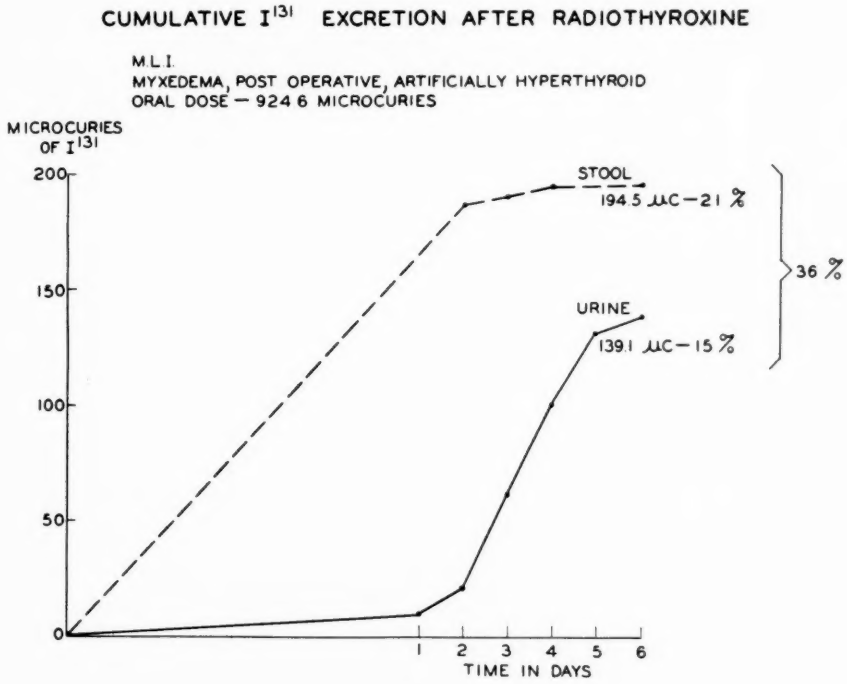

Fig. 1.

These patients were given oral "tracer" doses of less than 0.3 mg. of Na-l-thyroxine labeled with 585 to 924 microcuries of I^{131}. Quantitative I^{131} analyses were made on urine specimens collected every hour for six hours, then on twenty-four hour specimens for six days. Quantitative total fecal I^{131} assays of bile were performed at the same intervals. Protein-bound I^{131} determinations were performed two to four times during the six-day interval in four patients. The thyroxine came dissolved in 50 per cent propylene glycol. Our chromatographic analysis of the I^{131}-labeled Na-l-thyroxine showed that it contained less than 5 per cent free iodide.

Stool specimens were diluted with water and then thoroughly mixed in a Waring blender. The resultant volume was then measured. Tracerlab EU-5 chromium plated steel counting planchets were weighed. One milliliter aliquots were obtained from each specimen and placed in these planchets. Triplicate runs were made on each specimen. All samples were dried under an infrared lamp. These samples were then counted in a fixed geometry under a Tracerlab T.C.G. 1 Geiger tube within a two inch lead shield with a conventional scaling arrangement. All samples were counted for a period of time sufficient to insure a statistical error of less than 5 per cent.[7] The counting rate of each sample was compared to a prepared

standard which was in turn standardized by comparison to a long-life beta standard for iodine.[8] Correction for self-absorption was made based upon a prepared standard curve. Appropriate amounts of alkaline diluting fluid were added to insure even distribution and to prevent loss of I^{131} through volatilization. All values were corrected for decay to the day of administration of the I^{131}-labeled thyroxine. Serum protein-bound iodine I^{131} values were obtained by precipitation of protein by the method of Barker.[9] The precipitate was washed three times with distilled water and then resuspended. A one milliliter aliquot of this was counted for its beta activity.

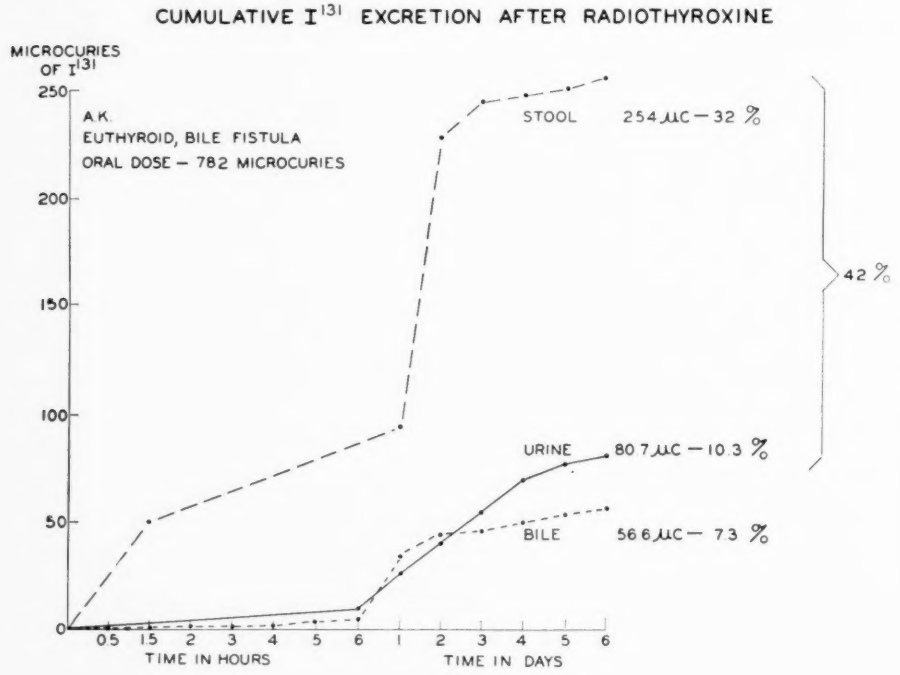

Fig. 2.

RESULTS

Figs. 1 to 4 show the data on each patient graphed. Separate bile, stool, and urine I^{131} excretions are plotted in cumulative total microcuries of I^{131}. Table I summarizes the most noteworthy features of this data on these four patients. Table II shows the blood protein-bound I^{131} levels in per cent per liter of blood for purposes of comparison of absorption of our compound with that used by Myant and Pochin.[6] It should be noted that two patients were studied six days, one seven days, and one four days. This does not seem to be of critical importance since the urinary, fecal and bile excretion tended to begin a plateau at four to five days.

Noteworthy is the qualitative similarity of cumulative urinary I^{131} excretion totaling 15.0, 10.3, 10.3, and 23.0 per cent of the administered dose in each patient, respectively. This is especially striking considering the diverse clinical thyroid states of the various subjects. Values of stool I^{131} excretion compare

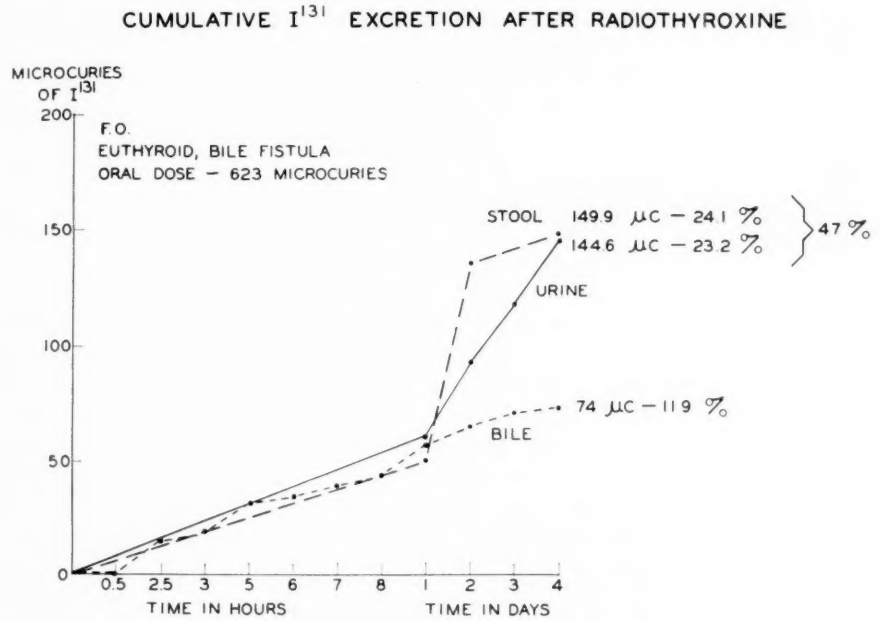
CUMULATIVE I131 EXCRETION AFTER RADIOTHYROXINE
MICROCURIES OF I131
200
150
100
50
0
F.O.
EUTHYROID, BILE FISTULA
ORAL DOSE – 623 MICROCURIES
STOOL 149.9 μC – 24.1 %
144.6 μC – 23.2 %
47 %
URINE
74 μC – 11.9 %
BILE
0.5 2.5 3 5 6 7 8 1 2 3 4
TIME IN HOURS
TIME IN DAYS

Fig. 3.

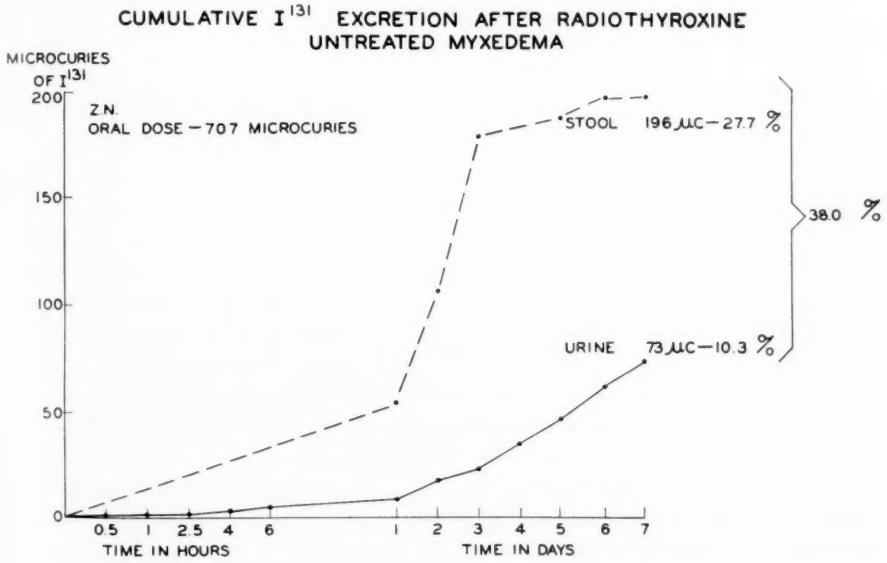
CUMULATIVE I131 EXCRETION AFTER RADIOTHYROXINE
UNTREATED MYXEDEMA
MICROCURIES OF I131
200
150
100
50
0
Z.N.
ORAL DOSE – 707 MICROCURIES
STOOL 196 μC – 27.7 %
38.0 %
URINE 73 μC – 10.3 %
0.5 1 2.5 4 6 1 2 3 4 5 6 7
TIME IN HOURS
TIME IN DAYS

Fig. 4.

TABLE I. SUMMARY OF TOTAL I^{131} EXCRETION IN URINE, STOOL, AND BILE IN FOUR PATIENTS GIVEN I^{131}-LABELED SODIUM-L-THYROXINE

PATIENT	M. L. I.	Z. N.	A. K.	F. O.
Clinical status	Artificial hyperthyroid	Untreated myxedema	Euthyroid bile fistula	Euthyroid bile fistula
Duration of study in days	6	7	6	4
Urine I^{131}, in per cent of administered dose	15	10.3	10.3	23
Stool I^{131}, in per cent of administered dose	21	27	32	24
Urine + stool I^{131}	36	37.3	42.3	47
Total bile I^{131}			7.3	11.9

even more closely at 21, 27, 32, and 24 per cent of the administered dose. Addition of stool and urine excretion values reveals a total of slightly more than one-third of the radiothyroxine derived I^{131} lost by these routes in each patient during this interval of about six days. Accumulative bile excretion in each of the two euthyroid patients with "bile fistulas" was similar in magnitude at 7.3 and 11.9 per cent of the administered dose. It is interesting to note that loss of I^{131} from the body through bile T-tube did not decrease the urine and stool excretion as compared with intact patients. Examination of Table II shows that our protein-bound I^{131} blood values exhibit a rise and fall similar in magnitude to those of Myant and Pochin after oral radiothyroxine. The absolute values in each patient, however, are also related to the specific activity of the radiothyroxine administered.

TABLE II. BLOOD I^{131} LEVELS FOLLOWING ORAL DOSES OF RADIOTHYROXINE. PROTEIN-BOUND I^{131} VALUES IN PER CENT OF ADMINISTERED DOSE PER LITER OF BLOOD

	½ HOUR	6 HOURS	24 HOURS	48 HOURS	96 HOURS	SPECIFIC ACTIVITY OF ADMINISTERED DOSE IN MICROCURIES PER 0.1 MG. OF THYROXINE
M. L. I. (Artificial hyperthyroidism)			0.3			306
Z. N. (Untreated myxedema)	1.0	3.1				416
A. K. (Euthyroid bile fistula)	0.8	3.9				440
F. O. (Euthyroid bile fistula)	11.0	15.0		11.0	7.5	1,249

DISCUSSION

Our doses of Na-l-thyroxine and of I^{131} radiation were not true tracer doses in that they were theoretically large enough to exert some metabolic effect. Our subjects were given somewhat less than 0.3 mg. of Na-l-thyroxine each, ranging from 0.3 mg. to 0.08 milligrams. This dosage of thyroxine has been found to be roughly equivalent[10] to 0.1 Gm. desiccated thyroid, which is in turn the daily requirement of thyroid in the majority of patients with myxedema.[11] It is unlikely that this increased quantity of thyroxine slightly above tracer-dose amounts significantly altered resultant data, however, since excretion of I^{131} by M. L. I., on 15 grains desiccated thyroid per day, was similar in magnitude to

euthyroids and to the patient with untreated myxedema not on thyroid. Five hundred to 900 microcuries of I^{131} may deliver enough radiation to the thyroid gland to decrease the rate of uptake of I^{131}. Clinically, this effect has not been observed, however, until a total of 500 microcuries has been administered to one person in 100 microcurie doses on five occasions.[12]

Theoretically, our data could have been altered in the two patients with "bile fistulas" by inhibiting the uptake of I^{131} in the thyroid by loading the thyroid with stable iodine derived from cholangiogram dye. However, the dye injected for cholangiography was removed in less than one minute by aspiration. The bile was then allowed to drain freely through the T-tube. It is probable that little iodine was absorbed from cholangiography because of the following factors: both patients had a stone in the common duct distal to the T-tube which caused obstruction complete enough to give bile recovery in quantities comparable to those recorded in the literature as representing total bile recovery;[13] the stools remained acholic; and the protein-bound iodine in F. O. was at the upper limits of normal, or 10.6 micrograms per cent, with inorganic iodine 8.0 micrograms per cent.

Obviously, no conclusions can be drawn about Na-l-thyroxine metabolism in naturally occurring clinical hyperthyroidism from the data obtained in our artificially hyperthyroid patient, since this patient had no functioning thyroid gland. Radioactive iodine metabolism would be expected to be different in this patient than in a patient with an overactive thyroid gland. The patient M. L. I. was of interest, however, because with artificial hyperthyroidism she simulated natural thyrotoxicosis in that her peripheral tissues were furnished thyroid hormone in excess of normal metabolic requirements. Yet she excreted Na-l-thyroxine derived I^{131} in stools and feces qualitatively similar to patients presumably delivering normal quantities of thyroid hormone to their peripheral tissues.

Evidence was presented in the introduction for and against poor absorption of thyroxine from the gastrointestinal tract. One explanation for the presence of thyroxine in the feces after an oral or intravenous dose of thyroxine is the hypothesis that thyroxine is actively excreted by the liver in the bile into the gut. Gross and LeBlond[14] demonstrated a large excretion of thyroxine by the liver into the gut when radioactive thyroxine labeled with large carrier doses of thyroxine was injected intravenously into rats. Doses containing one milligram of thyroxine were followed by the appearance of as much as 80 per cent of the iodine in the feces in twenty-four hours. Later work[15] suggested that this high fecal excretion may have been associated with large doses of thyroxine. Myant and Pochin[6] recovered only small quantities of I^{131}, 9 to 13 per cent of the administered dose at three days, in euthyroid human feces after oral or intravenous doses of 80 micrograms of thyroxine with 15 to 25 microcuries of I^{131}. In our studies the I^{131} excretion via the fecal route was approximately twice this value, but the dose of carrier thyroxine was larger than that of Myant and Pochin.[6] Still, our data support theirs qualitatively in that a relatively small portion of the original dose of thyroxine is recovered from the feces at three days. Our data were also comparable to theirs in that our protein-bound

iodine I^{131} blood levels approximated the same curve and quantitative range as their patients given radiothyroxine orally. We have gone a step further, however, by doing quantitative accumulative bile I^{131} excretions. These have shown that less than half as much I^{131} is recovered from the bile as from the feces in the first six days. The paucity of biliary excretion of thyroxine I^{131}, plus the fact that fecal excretion of thyroxine I^{131} was the same in both bile fistula (24 to 32 per cent of administered Na-l-thyroxine I^{131}) and in intact patients (21 to 27 per cent), indicates that thyroxine is present in the feces not because of high excretion by the liver via the bile into the gut, but either through lack of absorption from gut or active excretion through gut wall. It was noted in the introduction that the experiments of Myant and Pochin with I^{131}-labeled thyroxine given orally and intravenously[6] indicated that thyroxine was well absorbed from the gut when given orally. This evidence leaves the alternative of active excretion through gut wall into the gut as the most obvious explanation for the recovery of thyroxine I^{131} from the gut.

It should be emphasized, however, that the above experimental technique with radiothyroxine may not be sensitive enough to record significant differences between the metabolism of intravenously and of orally administered thyroxine. It is also difficult to be sure that the original l-thyroxine preparation has not become racemized to a significant extent during the experimental procedure. This might be of critical importance since d-thyroxine has been demonstrated to be less active metabolically than l-thyroxine.[16-18]

SUMMARY

Total six-day I^{131} excretion in urine and stool was similar in magnitude in one myxedematous, one artificially hyperthyroid, and two euthyroid adult humans after oral "tracer" doses of I^{131}-labeled Na-l-thyroxine.

Paucity of biliary excretion of thyroxine I^{131}, plus the fact that fecal excretion of thyroxine-derived I^{131} was the same in both bile fistula and in intact patients, indicated that thyroxine is present in the feces not because of fecal bile, but either through lack of absorption from gut or active excretion through gut wall. The fact that metabolism of intravenous thyroxine and oral thyroxine has been shown to be comparable suggests that thyroxine is actively secreted through the gastrointestinal wall into its lumen.

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THE EFFECT OF VARYING INSULIN DOSAGES ON THE RATE OF DECLINE OF THE BLOOD SUGAR IN THE MODIFIED GLUCOSE INSULIN TOLERANCE TEST

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THE term insulin sensitivity is properly used in the sense of the effect of insulin on the rate of withdrawal of glucose by the tissues from the extracellular fluid spaces. In a previous study[1] we introduced the modified Glucose Insulin Tolerance Test (G.I.T.T.) for the estimation of insulin sensitivity. This procedure consists of the intravenous injection of 25 Gm. of glucose followed at thirty minutes by 0.1 unit of crystalline insulin per kilogram of body weight. The degree of insulin sensitivity was stated as the rate of decline of the blood sugar concentration in milligrams per 100 ml. per minute calculated for the period of thirty minutes after insulin injection, which serves as a measure of the rate of withdrawal of glucose by the tissues.

Norgaard and Thaysen[2] previously attempted to measure insulin sensitivity by means of the "assimilation index" which was calculated from the decline of the blood sugar in the Insulin Tolerance Test (I.T.T.). However, it was found that the results obtained with this method are not comparable except when they are from the same individual, and even then only when the starting blood sugars are approximately the same.[3] Furthermore, when the blood sugar is reduced below the fasting level, counterregulatory mechanisms to the induced hypoglycemia are set in motion which influence the rate and extent of the fall of the blood sugar.[4] In an attempt to obviate these difficulties, Himsworth[3, 5] introduced the Glucose Insulin Tolerance Test which consists of the simultaneous administration of oral glucose and intravenous insulin. However, due apparently to variability in the rates of intestinal absorption of glucose, abnormal results are obtained with this procedure in many normal individuals.[1] These difficulties are thought to be overcome by the modified G.I.T.T., since both the glucose and insulin are administered intravenously, and the blood sugar level at thirty minutes after insulin is not low enough to stimulate counterregulatory mechanisms which might interfere with insulin action.

The insulin dosage utilized in the modified G.I.T.T. is that arbitrarily selected by Fraser, Albright, and Smith[6] for the I.T.T. The question may be posed as to whether the inferences as to insulin sensitivity derived from the modified G.I.T.T. are predicated on this particular insulin dosage. This objection was thought to be obviated by the findings of Somogyi[4] that the effect of insulin on the blood sugar level is not a stoichiometric one. Furthermore, Zucker and Berg[7] have shown that increasing the dose of insulin in dogs from 0.1 unit per kilogram to 1.0 unit per kilogram causes no increase in the rate of de-

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cline of the blood sugar level but only in the duration and intensity of insulin action. The explanation advanced for this phenomenon is that the rate of entry of glucose into the tissue and liver probably approaches a maximum even with small insulin dosages.[1] However, it has been shown in vitro[8] that the rate of glycogen formation by the rat diaphragm is within limits proportional to the concentration of insulin in the incubating medium.

Therefore, studies were undertaken of the rates of decline of the blood sugar concentration in the modified G.I.T.T. using varying insulin dosages. It was hoped, thereby, to clarify two questions: (a) whether any stoichiometry exists between insulin dosage and the rate of withdrawal of glucose from the circulation, and (b) at what dosage level will the maximum rate of decline of the blood sugar level in the modified G.I.T.T. be observed in nondiabetic individuals.

MATERIALS AND METHODS

Ten fasting nondiabetic individuals of average nutritional status were subjected to the intravenous Glucose Tolerance Test (G.T.T.) and subsequently to the modified G.I.T.T. For the G.T.T. 25 Gm. of glucose in 50 per cent solution were injected intravenously. Immediately before and at intervals of 30, 60, 120, and 180 minutes after the glucose administration duplicate capillary blood samples were collected for glucose determination. In carrying out the modified G.I.T.T. 25 Gm. of glucose in 50 per cent solution were given intravenously followed after thirty minutes by 0.1 unit of crystalline insulin per kilogram of body weight, intravenously. Duplicate capillary blood samples for glucose determination were withdrawn at fasting and at 30, 50, 60, 75, 90, 120, and 150 minutes.

In these same ten patients the modified G.I.T.T. was repeated at biweekly intervals substituting the following insulin dosages (1) 0.01 unit per kilogram, (2) 0.025 unit per kilogram, (3) 0.05 unit per kilogram, (4) 0.2 unit per kilogram, (5) 0.3 unit per kilogram. Since in the calculation of the rate of decline of the blood sugar it is the thirty minutes after insulin injection which is of importance, the blood samples were withdrawn only at fasting, 30, 50, and 60 minutes when these latter insulin dosages were used. This avoided the danger of hypoglycemia as the patients were given orange juice and sugar after the 60 minute specimen. Similarly higher insulin dosage ranges were not studied because of the danger of hypoglycemic shock. The blood sugar determinations were done by the Nelson modification of the Folin-Wu Micro-method.[9]

RESULTS

The criteria for a normal intravenous G.T.T. were a fasting capillary blood sugar value not higher than 140 mg. per cent, with a return of the 120 minute specimen to or below the fasting value.[10] The criteria for a normal modified G.I.T.T. using the standard insulin dosage of 0.1 unit per kilogram as previously established were a return of the blood sugar to fasting within 75 minutes after the injection of glucose and a rate of decline for the 30 minute period after insulin greater than 2.5 mg. per 100 ml. per minute.[1] All of our subjects met these norms. The mean blood sugar values for the modified G.I.T.T. in these ten individuals as compared to the G.T.T. are demonstrated in Fig. 1. The mean rate of decline for the G.T.T. was 2.2 ± 0.26 per 100 ml. per minute, while that for the modified G.I.T.T. using 0.1 unit per kilogram of insulin was 3.5 ± 0.28 mg. per 100 ml. per minute (Fig. 2).

The rates of decline of the blood sugar level $\pm$ one standard error of the mean* obtained with the various insulin dosages as compared to that in the G.T.T.

*Standard error of mean $= \sqrt{S(X-\bar{X})^2/N(N-1)}$ (14)

are also demonstrated in Fig. 2. The mean rate of decline in the insulin dosage range 0.01 unit per kilogram to 0.05 unit per kilogram varied from 2.4 mg. per 100 ml. per minute to 3.3 mg. per 100 ml. per minute. In the dosage range 0.05 unit per kilogram to 0.3 unit per kilogram the mean values for the rate of decline were almost identical. "t"* values were calculated for the differences in

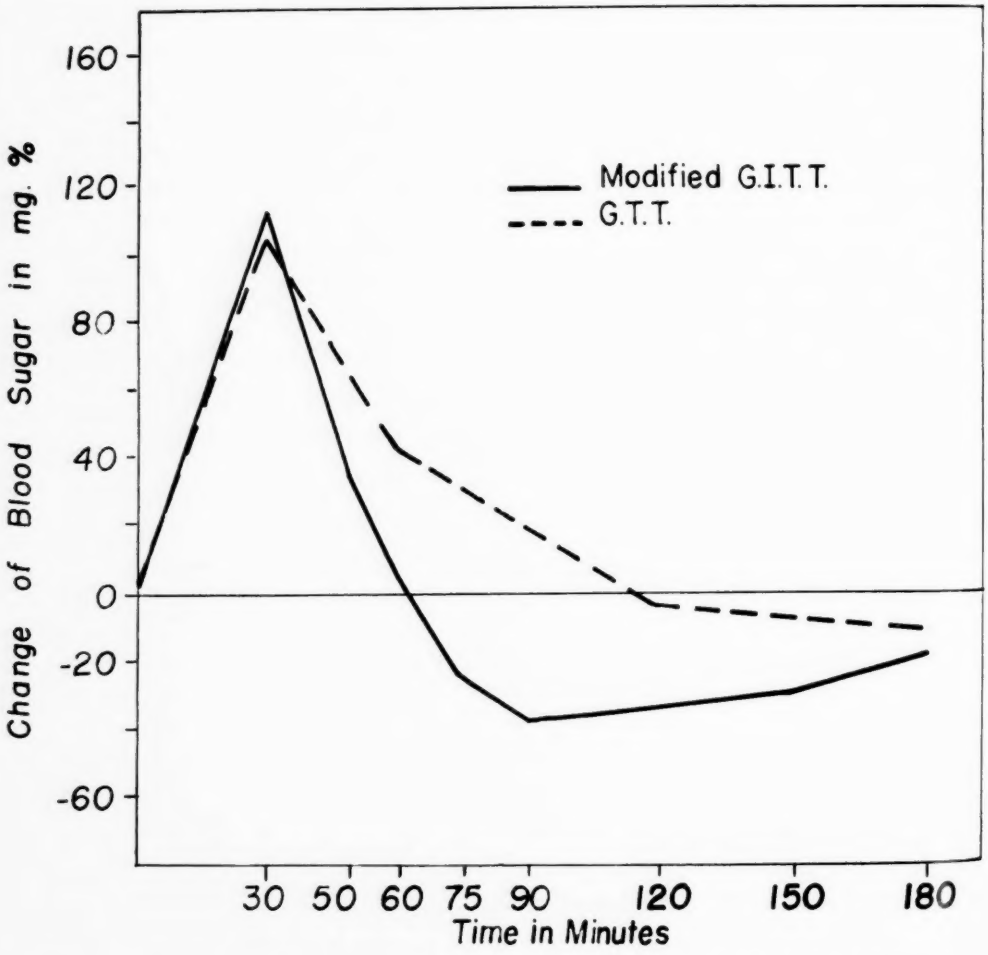

Fig. 1.—A comparison of the mean response of ten nondiabetic individuals to the G.T.T. and modified G.I.T.T. (0.1 unit of insulin per kilogram).

the rate of decline obtained with the various insulin dosages in the ten individual patients. It was found that the difference between 0 and 0.01 unit per kilogram was not significant. However, the differences between 0 and 0.025 and that between 0.025 and 0.05 unit per kilogram were significant at the 5 per cent level ($P<.05$). On the other hand the difference between the rates of decline obtained with 0.05 unit per kilogram and the higher insulin dosages was definitely not sig-

*Mean of differences/S.E. of mean difference.

nificant. This would indicate that the rates of decline gradually increased to a maximum with increasing insulin dosages up to 0.05 unit per kilogram, while further increase in insulin dosage up to 0.3 unit per kilogram causes no augmentation of the rate of decline of the blood sugar level.

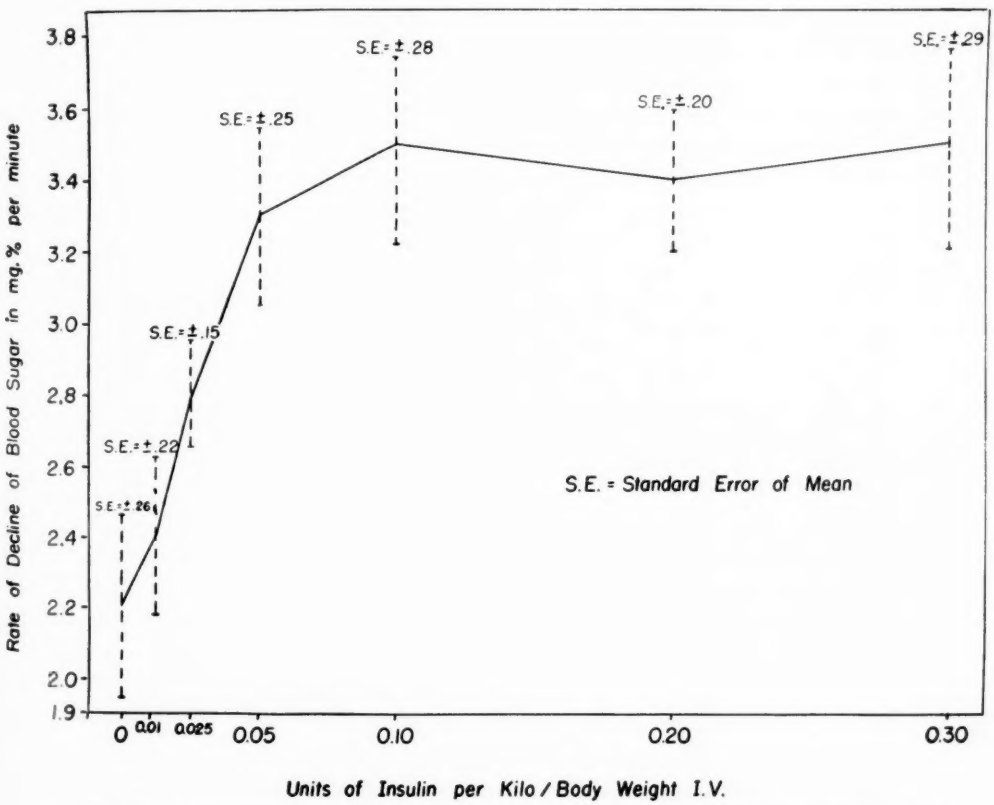

Fig. 2.—A comparison of the mean rate of decline of the blood sugar level for the 30 to 60 minute period of the modified G.I.T.T. ± one standard deviation in ten nondiabetic individuals after the intravenous administration of varying doses of insulin.

DISCUSSION

The results of this study support only in part the concept[4] that there is a lack of stoichiometry between insulin dosage and the rate of decline of blood sugar concentration. This is probably because the stoichiometry will be evident only at extremely low insulin dosage, i.e., a range of 0.7 unit (0.01 unit per kilogram) to 3.5 units (0.05 unit per kilogram) for the 70 kilogram individual. Above 3.5 units (0.05 unit per kilogram) up to 21 units (0.3 unit per kilogram) there is a complete lack of stoichiometry between insulin dosage and rate of decline, a plateau being observed graphically (Fig. 2).

These findings are in accordance with results recorded by others[7] who used these latter and higher insulin dosage ranges and who state that the rate of decline of the blood sugar level after intravenously injected insulin is not re-

lated to the dosage, increase in insulin dosage affecting only the depth and duration of hypoglycemia. The fact that the rate of decline reaches a plateau and is not further increased by a sixfold increase in insulin dosage supports the previously advanced theoretical concept that the rates of entry of glucose into the tissue and liver probably approach a maximum even with small insulin doses.[1] Furthermore, if the rate of decline of the blood sugar concentration is calculated in terms of the rate of disappearance of glucose from the blood sugar, a value of 460 mg. per kilogram per hour is obtained which corresponds well with the values obtained by Soskin and Levine[11] in the dog for the maximum rate of glucose uptake by the peripheral tissues, i.e., 550 mg. per kilogram per hour. This would also indicate that the chief site of glucose assimilation for the thirty minute period of the modified G.I.T.T. under consideration is the peripheral tissue rather than the liver. Support for this conclusion is lent by the work of Bondy and associates[12] who demonstrated that although the glucose output from the liver starts to decline almost immediately after insulin administration, the liver does not start to take up glucose for approximately forty-five minutes.

Previous workers using other procedures[5, 13] have attempted to use the difference between curves obtained after glucose administration alone and those obtained after glucose and insulin administration (i.e., the I/G ratio) as the criterion for insulin sensitivity. Similarly, an objection might be raised that the difference between the rate of decline in the G.T.T. and that in the modified G.I.T.T. would be a better index of insulin sensitivity than the rate of decline in the modified G.I.T.T. alone. However, since the rate of decline in the modified G.I.T.T. is a maximum, it is obvious that the patient with the best tolerance for glucose will have the least difference between the rates of decline in the two procedures and thus would be labeled as least sensitive to the action of insulin if the method of differences was used.

In view of these findings as to the response of the blood sugar to varying insulin dosages, it may be concluded that the dosage used originally in the modified G.I.T.T., namely, 0.1 unit per kilogram is probably the most satisfactory for the estimation of insulin sensitivity in the individual patient, since this dosage is just above the lowest at which a maximal response is observed. On the one hand, the use of a dose too low to give a maximal response would probably lead to variability in the results obtained in the normal individual. On the other hand, the use of large doses of insulin would probably overwhelm the mechanisms controlling insulin insensitivity and thus blur the significance of the results and hinder the identification of the insulin-insensitive patient.

It should also be emphasized that although in normal individuals the rate of decline of the blood sugar is not increased by insulin doses above 0.05 μ per kilogram it is expected that in insulin resistant diabetic patients as the insulin dosage is raised the rate of decline will increase until a maximum is obtained. Above this point further increase in insulin dosage should cause no change in the rate of decline. On the other hand, diabetic patients with normal insulin sensitivity should theoretically have a response identical with that of the normal.

SUMMARY

Studies on the rate of decline of the blood sugar level calculated for the 30 to 60 minute period of the modified G.I.T.T. were carried out on ten nondiabetic individuals using the following insulin dosages: (1) 0.01 unit per kilogram, (2) 0.02 unit per kilogram, (3) 0.05 unit per kilogram, (4) 0.10 unit per kilogram, (5) 0.20 unit per kilogram, (6) 0.30 unit per kilogram. It was found that the mean rate of decline was proportional to the insulin dosage up to 0.05 unit per kilogram. Further increase in insulin dosage up to 0.30 unit per kilogram caused no further acceleration of the rate of decline. From these observations it is concluded that 0.1 unit per kilogram is probably the most satisfactory dosage for use in the modified G.I.T.T. in the estimation of insulin sensitivity.

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FAILURE TO ACCOUNT QUANTITATIVELY FOR INFUSED INULIN IN HUMAN SUBJECTS

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QUANTITATIVE measurement of the extracellular fluid phase is essential to an understanding of disturbances in body water distribution. A series of three carbohydrates, sucrose,[1, 2] mannitol,[3, 6] and inulin[7, 9] have been extensively studied to measure the volume of the extracellular phase. These substances obviate the disadvantage of variable entrance of electrolytes in cells, diffuse into a smaller space of distribution, and presumably more accurately estimate the extracellular volume.

Inulin is reputed to have several advantages over either sucrose or mannitol: (1) It is believed that inulin is not metabolized to any appreciable degree and is rapidly and quantitatively recovered in the urine after intravenous injection.[10] This concept has been challenged recently in normal adult males,[11] nephrectomized dogs, and anuric patients.[12] (2) It is assumed to be distributed extracellularly since it does not penetrate the erythrocyte or escape through the normal renal tubules. (3) It is physiologically inert when properly prepared and exerts negligible osmotic pressure.

It possesses two disadvantages which render it unsuitable for use by a single injection method: (1) it diffuses slowly; (2) it is rapidly excreted by glomerular filtration.

In the use of inulin for the measurement of the extracellular fluid compartment it is required that the following conditions be fulfilled: (1) a constant extracellular fluid volume throughout the period of equilibration of inulin between plasma water and interstitial water; (2) uniform distribution of inulin between all compartments of the extracellular phase.

The inulin space can be measured in two ways. One method might be termed the "in-out" inulin space and the other the "infusion-recovery" space. In measuring the "in-out" space, one determines the total amount of inulin *infused* over the period of time required to attain equilibration and the total amount of inulin *excreted* in the urine during the period of infusion. Subtraction of the inulin *excreted* from that *infused* yields the amount of inulin *retained* at the time the infusion is discontinued. Dividing the amount of inulin retained by the plasma inulin level at the point of equilibration, one arrives at the volume

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of distribution. A prerequisite of this method is that no inulin be metabolized. If an unknown degree of metabolism of inulin occurred, the amount of inulin calculated as retained would be falsely high, with a resultant erroneously high inulin space.

A second method of measuring the inulin space is by the "infusion-recovery" method. Inulin is infused to the point of equilibration, at which time the infusion is discontinued and all the urine excreted in the succeeding twenty-four hours is quantitatively collected. The quantity of inulin recovered in the postinfusion urine divided by the plasma concentration of inulin at the point of equilibration yields the volume of distribution of inulin. It is generally accepted that inulin is quantitatively excreted in the urine within a twenty-four hour period in normal human subjects following parenteral administration.[10]

It is reported that the volume of distribution of inulin in man ranges from 15 to 16 per cent of body weight, and is not increased if the equilibration time is prolonged from six through twenty hours.[8] During routine measurements of twenty-four hour glomerular filtration rates in nonedematous subjects, we observed that inulin spaces seem to progressively *increase* as measured by the "in-out" method. It was further noted that in the same individual, inulin spaces measured by the "infusion-recovery" method after a twenty-four hour infusion were significantly smaller than either six or twenty-four hour "in-out" spaces. These findings proivde the basis of the present paper.

METHODS

Nonedematous adult male patients free of renal or hepatic disease served as subjects for this investigation. The subjects were allowed food and water ad libitum, but were kept recumbent in bed throughout the experimental procedure. No preliminary hydration was employed. Prior to infusion, blood samples and twenty-four hour urine samples were obtained for determination of plasma and urine blanks, respectively. A priming injection of inulin was followed by a constant sustaining infusion for twenty-four hours. Plasma levels of inulin were maintained at about 20 mg. per cent. All solutions were prepared in 0.45 per cent saline solution. A Bowman constant injection pump delivered the solutions at an average rate of 1.9 ml. per minute, with a mean variation of 3.5 ± 2.5 per cent in the volume flow per minute. Quantitative urine collections were effected by means of an indwelling multi-eyed urethral catheter. The bladder was rinsed with three 20 ml. portions of saline solution upon completion of a given urine-collection period to effect quantitative recovery of inulin. Blood samples were drawn at the end of a given inulin space determination. Urine was collected during and for some three to four days following the inulin infusion.

The total amount of inulin injected was computed in two ways. In one group of patients the inulin "in" was estimated by multiplying the pump rate by the concentration of inulin in the infusate. In a second group of patients a measured volume of inulin of known concentration was alloted for a given experiment and the amount which was not infused was simply subtracted from the total to determine the precise amount of inulin which went into the patient. Inulin in plasma and urine was determined by the methods of Harrison[13, 14] and/or Roe and Epstein.[15] Inulin recoveries from plasma and urine by the Harrison method averaged 99.4 ± 3.2 per cent and 98.3 ± 2.5 per cent, respectively, whereas by the Roe and Epstein method they averaged 99 ± 2.9 per cent and 100.1 ± 2.8 per cent, respectively.

To arrive at the amount of inulin infused, the concentrations of inulin in the infusate and in the original ampules of 10 per cent inulin were determined. To exclude the possibility

TABLE I. AMOUNT OF FERMENTABLE MATERIAL IN AMPULES OF 10 PER CENT INULIN

LOT NO.	INULIN CONTENT PRIOR (A) TO YEASTING (MG. PER CENT)	INULIN CONTENT AFTER FERMENTATION FOR 1 HR. WITH 20 PER CENT YEAST		
		(B) (MG. PER CENT)	(A-B) (MG. PER CENT)	FERMENTABLE MATERIAL (PER CENT)
204A1	8,750	8,573	177	2.0
201A1	8,320	8,238	82	1.0
202A2	8,300	8,217	83	1.0
203A1	8,520	8,337	183	2.1
Mean			131	1.5
Standard deviation			±56	±0.6
Probability value			0.01	--

of significant amounts of fructose or other fermentable material in the 10 per cent inulin solution being considered as inulin, 10 per cent inulin solutions were analyzed by the Harrison method before and after yeast fermentation. The yeasting procedures employed had previously been shown to destroy up to 300 mg. per cent of glucose. As can be seen from Table I, the fraction of fermentable noninulin material (1.5 ± 0.6 per cent) present was well within the analytic error of the inulin method. It would appear however that a statistically significant (P = 0.01) although admittedly small amount of fermentable noninulin material was present in the various lot numbers analyzed. All values for inulin infused were therefore corrected for the fraction of fermentable substance which it was assumed would be metabolized in the body.

RESULTS

The effects of the duration of the infusion on the size of inulin "in-out" spaces in nonedematous subjects are summarized in Table II. It can be seen that during six to eight hours of infusion, the mean inulin spaces ranged from 13.5 to 14.1 liters per 1.73 square meters. After eighteen to twenty-four hours of infusion the inulin spaces seemed to increase to a mean of 17.9 to 21.7 liters per 1.73 square meters. Inulin spaces determined by the infusion-recovery

TABLE II. EFFECT OF DURATION OF INFUSION ON THE SIZE OF THE INULIN "IN-OUT" SPACE IN NONEDEMATOUS SUBJECTS

SUBJECT	"IN-OUT" INULIN SPACES AT VARIOUS INTERVALS DURING CONTINUOUS INULIN INFUSIONS (L./1.73 SQ. M.)						B-A* (L./1.73 SQ. M.)	URINE RECOVERY SPACE (L./1.73 SQ. M.)†
	6 HR.	7 HR.	8 HR.	18 HR.	19 HR.	24 HR.		
1. A. B.§	13.0	13.3	13.5	--	--	26.1	+12.8	10.7
2. J. C.§	16.4	13.3	13.6	--	--	21.0	+ 6.6	12.3
3. J. W.§	11.8	12.3	13.4	--	--	21.0	+ 8.5	10.7
4. J. T.‖	14.7	15.3	--	29.4	27.2	--	+13.3	11.8
5. H. W.‖	9.8	9.4	--	14.7	15.5	--	+ 5.5	7.9
6. A. L.‖	20.2	23.5	--	20.7	17.2	27.1	- 0.1	11.7
7. K. D.‖	10.6	11.7	--	11.2	11.8	13.2	+ 1.0	9.9
Mean	13.8	14.1	13.5	19.0	17.9	21.7	+ 6.8‡	10.7
Standard deviation	±3.6	±4.5	±0.1	±8.0	±6.6	±5.5	± 5.5	±1.49

*A = mean of 6,7,8 hour spaces.
B = mean of 18,19,24 hour spaces.
†Inulin space based on amount of inulin recovered in urine 24 hours post-infusion.
‡Mean difference of +6.8 L./1.73 sq. m. is statistically significant (P = 0.01).
§Amount of inulin "in" estimated from pump rates.
‖Amount of inulin "in" based on actual volumes infused.

TABLE III. RECOVERY OF INULIN FROM THE URINE IN NONEDEMATOUS SUBJECTS FOLLOWING TWENTY-FOUR HOURS OF CONTINUOUS INFUSION

SUBJECT	INULIN RETAINED* (GM.)	INULIN EXCRETED DURING FIRST 24 HR.-POST INFUSION (GM.)	INULIN UNACCOUNTED FOR (GM.)	INULIN UNACCOUNTED FOR CALCULATED AS PER CENT OF INULIN INFUSED
1. V. S.‡	4.61	2.06	2.55	6.05
2. H. B.‡	4.60	2.46	2.14	5.39
3. A. B.‡	5.66	2.23	3.43	8.45
4. J. C.‡	3.77	1.90	1.87	5.21
5. J. W.‡	5.39	2.66	2.73	6.81
6. J. T.§	7.06	3.05	4.01	15.20
7. H. W.§	6.10	3.10	3.00	6.64
8. A. L.§	9.37	4.01	5.36	9.03
9. K. D.§	5.29	3.98	1.31	2.38
		Mean	2.94†	7.24
		Standard deviation	±1.21	±3.55

*Inulin retained = Inulin "in"—Inulin "out" *during* infusion.
†Mean difference of 2.94 grams is statistically significant (P = <0.01).
‡Amount of inulin "in" estimated from pump rates (IV).
§Amount of inulin "in" based on actual volumes infused.

method yielded a mean value of 10.7 liters per 1.73 square meters which is comparable to those published by Levitt and associates,[9] but is appreciably lower than those obtained by the "in-out" method.

These data prompted us to determine whether inulin could be quantitatively recovered from the urine following prolonged intravenous infusions. Table III summarizes data on the urinary recovery of inulin following twenty-four hours of continuous infusion. It can be seen that a mean of 2.94 ± 1.21 ($P < 0.01$) Gm. of inulin was unaccounted for. This amount of inulin was equivalent to 7.2 per cent of the inulin infused. In another series of experiments which were designed to reproduce the findings of Levitt and associates,[9] inulin was infused for only six hours. From Table IV it can be seen that on the average only 0.32 ± 0.35 ($P = 0.1$) Gm. of inulin were unaccounted for under the circumstances of a shorter infusion. This was equivalent to roughly 2.7 per cent of the amount of inulin retained as calculated by the "in-out" method. It would appear that the amount of inulin unaccounted for increased in direct proportion

TABLE IV. RECOVERY OF INULIN FROM THE URINE IN NONEDEMATOUS SUBJECTS FOLLOWING SIX HOURS OF CONTINUOUS INFUSION

SUBJECT	INULIN RETAINED* (GM.)	INULIN EXCRETED DURING FIRST 24 HR.-POST INFUSION (GM.)	INULIN UNACCOUNTED FOR (GM.)	INULIN UNACCOUNTED FOR CALCULATED AS PER CENT OF INULIN INFUSED
1. L. L.‡	2.39	2.06	0.33	3.03
2. J. F.‡	3.06	2.14	0.92	7.75
3. E. P.‡	2.44	2.39	0.05	0.44
4. J. J.‡	2.65	2.43	0.22	1.80
5. W. A.‡	1.95	1.86	0.09	0.80
		Mean	0.32†	2.76
		Standard deviation	±0.35	±2.97

*Inulin retained = Inulin "in" – Inulin *"out" during infusion.*
†Mean difference of 0.32 gram is not statistically significant (P = 0.1).
‡Amount of inulin "in" estimated from pump rates (IV).

TABLE V. URINE INULINOID BLANKS BEFORE AND AFTER 24 HOUR INULIN INFUSIONS

SUBJECT	PREINFUSION (A) (MG./24 HR.)	POSTINFUSION INULINOID EXCRETION (MG./24 HR.) 24-48 HR. (B)	48-72 HR.	72-96 HR.	B-A
1. V. S.*	306	277	---	---	− 23
2. H. B.*	331	296	---	---	− 35
3. A. B.*	148	159	---	---	+ 11
4. J. C.*	155	234	---	---	+ 79
5. J. W.*	91	180	---	---	+ 89
6. J. T.†	84	340	---	---	+256
7. H. W.†	43	268	---	---	+225
8. A. L.†	219	457	165	107	+238
9. K. D.†	57	217	171	90	+160
				Mean	+111
				Standard deviation	±114

*Inulin method—Harrison's modification of method by Alving and associates.
†Inulin method—Roe and Epstein.

to the duration of the infusion. Blank determinations of "inulin" in urine before and after twenty-four hour inulin infusions are summarized in Table V. Harrison's method yielded data indicating that there was quantitative excretion of inulin within the first twenty-four hours post infusion. By the method of Roe and Epstein, it would appear that the urine blank values were somewhat lower and urine specimens twenty-four to forty-eight hours post infusion contained an appreciable amount of inulin of the order of magnitude of approximately 200 milligrams. In two patients (A. L. and K. D.), the urine collections were continued beyond twenty-four hours post infusion. The urine blanks returned to the preinfusion level in one subject (A. L.) within forty-eight to seventy-two hours after infusion, and in the other (K. D.) within seventy-two to ninety-six hours post infusion. This would appear to rule against the possibility that the unaccounted-for inulin (of the order of magnitude of some 3,000 mg.) could be slowly and progressively excreted over many days.

Consideration was given to the possibility that the apparent increase in inulin spaces with continued infusions might be due to derangements in fluid balance. Fluid intake estimates included infused inulin and all other fluids ingested. No estimate was made of food water or of water loss through in-

TABLE VI. COMPARISON OF FLUID INTAKE VS. URINE OUTPUT DURING TWENTY-FOUR HOUR INFUSIONS IN NONEDEMATOUS SUBJECTS

SUBJECT	FLUID INTAKE* (LITERS/24 HR.)	URINE OUTPUT (LITERS/24 HR.)	INTAKE-OUTPUT (LITERS)
1. A. B.†	4.33	2.82	+1.51
2. J. C.†	5.46	5.07	+0.39
3. J. W.†	5.16	3.34	+1.82
4. J. T.‡	3.97	3.35	+0.62
5. H. W.‡	4.53	2.42	+2.11
6. A. L.‡	3.95	2.42	+1.53
7. K. D.‡	3.90	2.22	+1.68
Mean	4.47	3.09	+1.38
Standard deviation	---	---	±0.63

*Fluid intake = Inulin solution infused + other fluids (water, tea, coffee, milk, etc.).
†Inulin solution volume "in" estimated from pump rates.
‡Inulin solution volume "in" based on actual volumes infused.

sensible routes. From Table VI it can be seen that, although the subjects were in positive water balance, the volumes were insufficient to account for the increases in inulin space observed with continued infusions.

DISCUSSION

The amount of inulin "loss" following twenty-four hours of continuous infusion seems to be related to the duration of the infusion, since the results obtained during the course of these investigations suggest that a small but statistically significant amount of inulin infused over a twenty-four hour period is not quantitatively excreted in the urine. The question is whether these results represent a physiologic phenomenon or can they simply be ascribed to analytic or technical errors. The error in estimating the amounts of inulin infused due to varying the pump rates can be disregarded, since similar results were obtained using a measured volume of inulin solution. It is unlikely that the amounts of inulin unaccounted for were entirely due to analytic error, since one would have to postulate a constant error in either inulin infusate analyses or urinary inulin analyses to account for the consistent amount of inulin "lost" in each experiment. One would expect the analytic errors to be random in distribution.

The fact that the amount of inulin "lost" seems to vary directly with the duration of the infusion further supports the thesis that inulin is being "lost" somewhere in the body. Recently Finkenstaedt and associates[12] reported that in nephrectomized dogs and anuric patients, serum inulin levels fell continuously with progressive increases in the apparent "inulin spaces." They concluded that inulin could not be used to measure extracellular space in anuric subjects. The failure of inulin to equilibrate was unexplained but might have been due to catabolism, transfer into cells, entry into the reticuloendothelial system or other compartments, in the opinion of Finkenstaedt and associates.[12] We are unable to say at this point where or how a fraction of inulin is disposed. Significant loss in intestinal juices seems unlikely. Metabolism or storage by the liver or other tissues must be considered. It is of interest to note that dextrans, which, like inulin, are long-chain polysaccharides, have recently been shown to be metabolized in the human being.[16, 17]

The evidence presented by these studies to the effect that inulin is either lost or metabolized in the body would preclude the use of this solute for the measurement of extracellular volume by the "in-out" method, since it is essential that the solute not be metabolized. Since the recovery of inulin from the urine after an infusion requires some twenty-four hours, it is conceivable that a significant amount of inulin may be "lost" during this period and therefore inulin spaces based on this approach might also be suspected.

CONCLUSIONS

1. A small but statistically significant fraction of injected inulin cannot be recovered in the urine following twenty-four hour infusions in normal adult male subjects.

2. The failure to quantitatively account for infused inulin precludes the use of this solute for the measurement of the extracellular volume by the "in-out" method.

3. The possibility of metabolism of a fraction of retained inulin during its slow excretion over a twenty-four to forty-eight hour period must be considered in the determination of inulin spaces by the "infusion-recovery" method.

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IDIOPATHIC CONGENITAL DILATATION OF THE PULMONARY ARTERY

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THE clinical diagnosis of idiopathic congenital dilatation of the pulmonary artery has, until recently, been difficult to establish. With the development of cardiac surgery differentiation of this anomaly from those defects associated with pulmonary artery dilatation which are amenable to surgical correction has become important. Through the use of cardiac catheterization, criteria for the antemortem diagnosis of this lesion have been elaborated, making possible a more precise definition of the clinical frequency, prognosis, and sequelae of this anomaly. The present report consists of six catheterized cases to draw attention further to this clinical entity.

MATERIAL

The six cases reported were referred to the Cardiovascular Department of Michael Reese Hospital for further study for congenital heart disease. All patients had a complete clinical examination, including history, physical examination, hemogram, fluoroscopic and x-ray examinations, electrocardiogram, right heart catheterization, and respiratory studies (see Tables I, II, and III).

OBSERVATIONS

Full clinical data on each patient are given in the appended case reports. Only the pertinent and important data will be summarized.

(a) *History.*—None of the patients had sought medical advice because of any symptoms referable to the cardiopulmonary system. Rather, congenital heart disease had been diagnosed because of murmurs found incidentally on routine or unrelated examinations; but two of the patients, on questioning, reported minor cardiorespiratory symptoms. Thus, one of them (L. P.) complained of transitory dyspnea and fatigue on one previous occasion, apparently related to an upper respiratory infection. The other patient (J. D.) also complained of exertional dyspnea easily modified by her emotional state.

None of the patients gave a history of cyanosis or of rheumatic fever, scarlet fever, diphtheria, syphilis, or tuberculosis.

(b) *Physical Findings.*—None of the patients had cyanosis, clubbing, chest deformities, retardation of growth or development, abnormal peripheral pulses, or signs of congestive heart failure. In fact, the only abnormalities found on examination were related to cardiac auscultation and palpation. In each case, the second pulmonic sound was accentuated and was greater than the second aortic. A systolic murmur was heard with maximum intensity in

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TABLE I. SUMMARY OF RESTING OXYGEN CONTENT (VOL. PER CENT)

	IVC	SVC	HIGH RA	MID RA	LOW RA	LOW RV	MID RV	HIGH RV	MPA	RPA	LPA
J. H.	-	11.2	12.3	13.3	13.0	14.0	13.7	13.5	14.0	-	13.5
D. R.	-	14.4	14.6	15.8	-	-	15.4	14.6	15.4	15.4	-
S. J.	12.6	12.3	12.5	12.5	-	12.2	-	11.6	12.5	12.3	-
D. F.	12.5	13.2	14.4	-	14.1	14.0	14.0	-	-	14.1	13.8
L. P.	-	13.3	13.0	13.6	13.5	13.5	14.0	14.2	14.2	13.9	-
J. D.	14.3	12.9	13.7	14.2	13.6	13.7	13.3	14.1	14.0	-	13.8

IVC = inferior vena cava.
SVC = superior vena cava.
RA =right auricle.
RV = right ventricle.
MPA = main pulmonary artery.
RPA = right pulmonary artery.
LPA = left pulmonary artery.

TABLE II. SUMMARY OF PRESSURES (MM. HG.) AT REST AND UPON EXERCISE

	REST						EXERCISE			
	MPA S/D	RV S/D	RA MEAN	RPA MEAN	MPA MEAN	BA MEAN	RV MEAN	RPA MEAN	MPA MEAN	BA MEAN
J. H.	12/3	22/3	0	9	8	88	27/2	-	12	-
D. R.	17/9	21/-1	2	12	11	79	-	-	13	-
S. J.	22/8	23/2	3	18	16	70	-	23	-	-
D. F.	20/8	19/2	3	14	14	95	-	14	-	-
L. P.	30/9	30/3	4	27	24	86	-	-	30	88
J. D.	19/8	20/2-5	1	13	13	94	-	-	13	-

BA = brachial artery.
S/D = systolic/diastolic.
See Table I for meaning of other symbols.

TABLE III. SUMMARY OF OTHER PERTINENT DATA

	OXYGEN SATURATION OF BRACHIAL ARTERY (%)		A-V DIFFERENCE (VOL. %)		O_2 COSUMPTION (C.C./MIN./M.^{2})		CARDIAC INDEX (L./MIN./M.^{2})		VENTILATION (L./MIN./M.^{2})		VENTILATION EQUIVALENT	
	REST	EXER.	REST	EXER.	REST	EXER.	REST	EXER.	REST	EXER.	REST	EXER.
J. H.	96	96	2.2	4.0	100	291	4.5	7.3	5.05	10.13	50	35
D. R.	95	95	3.0	4.2	126	140	4.2	3.4	5.27	5.55	42	40
S. T.	95	95	3.7	6.4	133	358	3.6	5.6	5.11	12.68	38	35
D. F.	97	96	3.1	5.3	127	322	4.1	6.1	6.74	8.77	53	27
L. P.	99	97	5.5	4.6	156	379	2.8	8.2	3.69	8.96	23	24
J. D.	93	95	4.0	6.1	102	315	2.6	5.2	2.87	19.79	28	62

Exer. = exercise.

the second or third intercostal space to the left of the sternum. A thrill sometimes could be palpated in the same location. The only constant feature of this murmur was its very inconstancy. The classifying characteristics of a murmur, i.e., its intensity, its quality, its pitch, its transmissibility, and its alteration with position and phase of respiration, would change from time to time and show extreme variations in a given patient. In addition, a short inconstant diastolic murmur in the pulmonic region was heard in three patients.

(c) *Fluoroscopic and X-ray Examination.*—Final judgment about the cardiac silhouette and the lung fields was arrived at only after correlating both methods of examination. In all cases, the pulmonary artery and its main branches were large and dynamic, as is best seen in the posteroanterior and right

anterior oblique views (Figs. 1, 2, 3, and 4). Expansile pulsations ("hilar dance") were not seen. Peripheral vascular markings were normal in each instance. The discrepancy between large hilar vessels and diminished or absent peripheral vascular markings, as seen generally in "isolated" pulmonary stenosis with poststenotic dilatation, was not present in our cases.

No cardiac chamber enlargement was seen, and the aortic knob was normal.

(d) *Electrocardiogram.*—The electrocardiogram was within normal limits in all patients. In four of them the "electrical" position was vertical, being semihorizontal in the other two. Except for sinus arrhythmia, no irregularities of rhythm were noted.

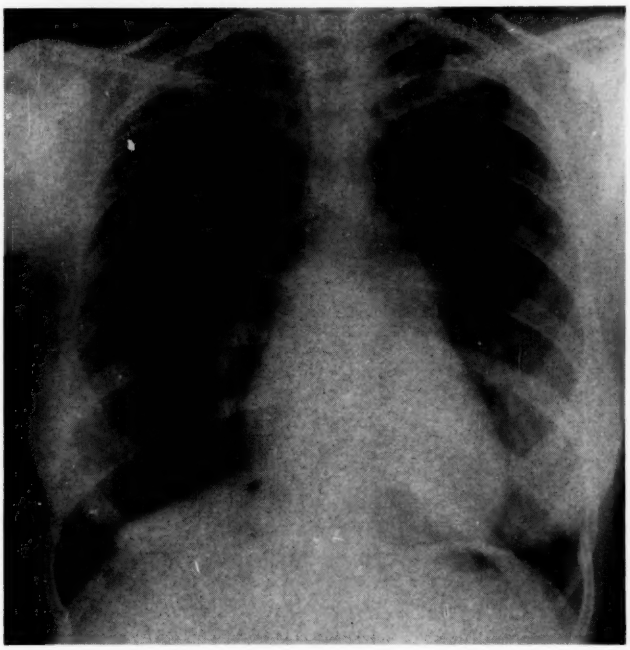

Fig. 1.—Posteroanterior view of chest in J. H.

(e) *Laboratory Data.*—Hemogram, urinalysis, and blood Kahn were normal in all patients.

(f) *Cardiac Catheterization.*—As can be seen from Table I, there was no evidence of a left to right shunt. The arterial oxygen saturations were normal in all cases, both at rest and on exercise, indicating the absence of a right to left shunt.* As the catheter was withdrawn from the pulmonary artery into the right ventricle, continuously recorded pressures showed a normal systolic pressure gradient; in one case (J. H.) there was a slightly increased gradient, but

*It is remotely possible that a tiny shunt of no significance might have been present and was not detected by catheterization. It would be difficult to see how this minor shunt could have led to dilatation of the pulmonary artery, even if present.

the right ventricular pressure was normal.* Pulmonary artery pressures were normal in all but one instance (L. P.) in which there was a slight elevation. A small increase in pulmonary artery pressure occurred on exercise in three patients. However, this elevation (3 to 6 mm. Hg, mean) was of a degree occasionally seen in normal subjects both in our experience and that of others.[1, 2, 3]

Cardiac output and cardiac index values were normal in five patients, both at rest and on exercise. In one (D. R.), whose resting output was at the upper limit of normal, there was a decrease during exercise. This is a well-recognized occurrence when the patient experiences anxiety at rest.

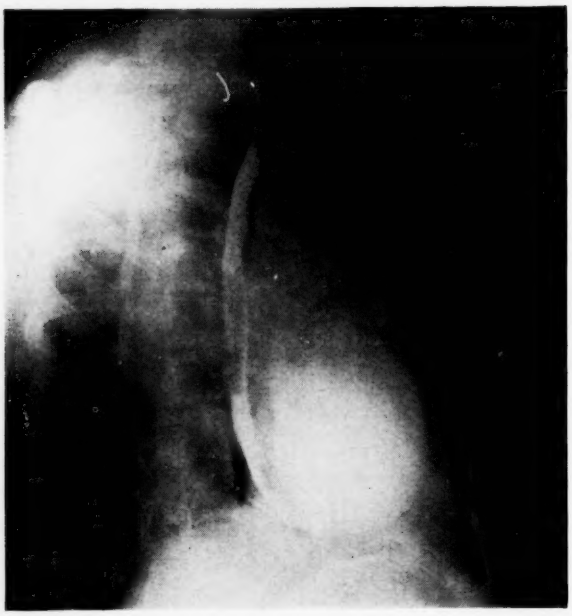

Fig. 2.—Right anterior oblique view of chest in J. H.

The Valsalva maneuver was performed by four patients; the normal response of an arterial blood presure "overshoot," followed by a reflex bradycardia, occurred in three. The absence of the overshoot in the last case is ascribed to an alteration in the autonomic nervous system function.[4]

(g) *Respiratory Studies* (Compare Table III).—Oxygen consumption at rest, measured as part of the output determinations, was within normal limits in all instances. Except in one case (D. R.), it approximately doubled on a standard type of mild exercise, which is the response obtained from normal subjects.[5] The ventilatory equivalent (liters ventilated per 100 ml. of oxygen consumed) on exercise was within the normal limits observed in this laboratory[5] in all but one of the cases. The rise on exercise which occurred in one of the patients (J. D.) is unexplained.

*The presence of a dynamically significant pulmonic stenosis can thus be discounted, especially since such small pressure gradients can be explained on the basis of the pulmonary artery segment dilatation.

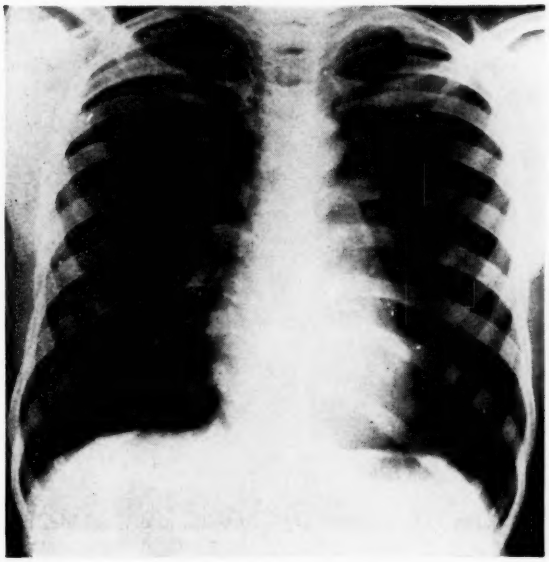

Fig. 3.—Posteroanterior view of chest in D. F.

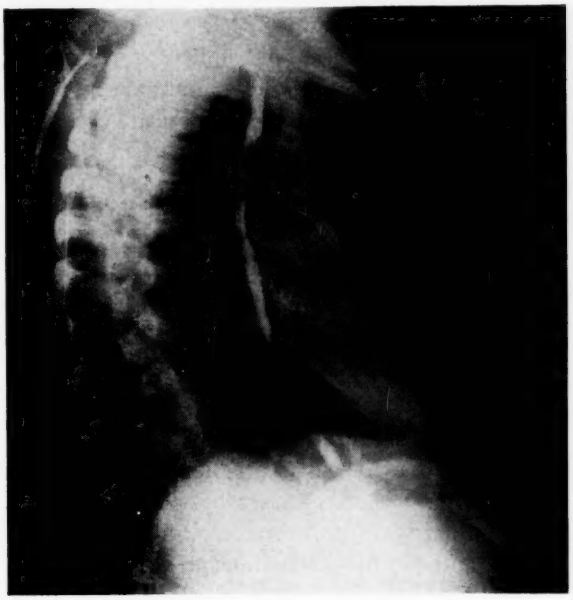

Fig. 4.—Right anterior oblique view of chest in D. F.

TABLE IV. A DIGEST OF THE CLINICAL FEATURES AND DIFFERENTIAL

	HISTORY	MURMURS	THRILLS
Idiopathic congenital dilatation of the pulmonary artery	No symptoms except rarely exertional dyspnea	Inconstant systolic, maximum in left 3rd I.C.S.; short diastolic may be present	Usually present in left 3rd I.C.S., but may be transient
"Isolated" pulmonary stenosis	Usually symptomatic but may be without symptoms	Systolic in 2nd or 3rd left I.C.S.	Present in 2nd or 3rd left I.C.S.
Atrial septal defect	Majority are dyspneic with exertion; may be asymptomatic	Systolic at base; diastolic may be present	May be present
Interventricular septal defect	Absence of, few or many symptoms	Systolic in left 3rd or 4th I.C.S.; rarely a diastolic	Usually present
Patent ductus arteriosus	Absence of, few or many symptoms	Typical machinery murmur in pulmonic area in 95% of cases*	Present in 50% of cases*
Aortic-pulmonary septal defect	May have absence of, few or many symptoms	Systolic and diastolic, may or may not be continuous in 2nd and 3rd I.C.S., occasionally systolic alone.	Usually present

I.C.S. = intercostal space.
*=(9).
†=(10).
‡=(11).

DISCUSSION

Greene and associates[6] have reviewed the literature of the last thirty years on idiopathic congenital dilatation of the pulmonary artery and established the following criteria for its anatomical diagnosis: (1) simple dilatation of the pulmonary trunk with or without involvement of the rest of the arterial tree; (2) absence of intra- or extracardiac shunts; (3) absence of chronic cardiac or pulmonary disease; (4) absence of arterial disease, such as syphilis or more than minimal atheromatosis or arteriosclerosis of the pulmonary vascular tree. Grishman and co-workers[7] similarly placed the main emphasis of diagnostic criteria on the absence of primary hemodynamic disturbances to which the pulmonary artery dilatation might be secondary.

From a clinical standpoint there are several findings which aid in diagnosis. As outlined in Table IV these are: (1) a systolic murmur (and thrill) loudest in the second or third intercostal space to the left of the sternum, the outstanding characteristic of which is its inconstancy; (2) an accentuated pulmonary second sound; (3) a normal electrocardiogram; (4) a large pulmonary artery with prominent major branches, but normal vascularity of the peripheral lung fields; and (5) otherwise normal cardiac silhouette. The diagnosis should not be restricted to those cases which show concomitant hypoplasia of the aorta.[8]

In the clinical differential diagnosis of this entity, one should consider such other acyanotic congenital anomalies as isolated pulmonary stenosis,

DIAGNOSIS OF IDIOPATHIC CONGENITAL DILATATION OF THE PULMONARY ARTERY

SECOND PULMONARY SOUND	SYSTEMIC BLOOD PRESSURE	ELECTROCARDIOGRAM	X-RAY AND FLUOROSCOPIC EXAMINATIONS
Accentuated	Normal	Normal	Large pulmonary artery and branches with normal peripheral vascularity of lungs
Diminished, normal or accentuated	Normal	Usually right heart strain or right bundle branch system block	May have post-stenotic dilatation of pulmonary artery, but peripheral vascularity of lungs is diminished
Usually not accentuated; may be split	Normal	Right bundle branch system block as a rule†	Large pulmonary artery with increased vascularity of lung fields; expansile pulsations (''hilar dance'') may be present
Accentuated	Generally normal; rarely with aortic insufficiency, a large pulse pressure present	Normal, left axis shift or left heart strain; large diphasic QRS complexes common‡	Usually normal, but may show large pulmonary artery with increased vascularity of lung fields
Accentuated	Large pulse pressure usually	Normal, non-specific abnormalities or left heart strain; first degree A-V block found frequently	Large pulmonary artery, increased vascularity of lung fields, dynamic aorta with prominent knob, expansile pulsations (''hilar dance'')
Accentuated	Large pulse pressure	Left heart strain	Large pulmonary artery, increased vascularity of lung fields, dynamic aorta with prominent knob, expansile pulsations (''hilar dance'')

atrial septal defect, interventricular septal defect, patient ductus arteriosus, and aortic-pulmonary septal defect.

As stated previously, patients with congenital idiopathic dilatation of the pulmonary artery have no complaints except exertional dyspnea on rare occasions. Thus, the presence of symptoms should make one immediately consider one of the other congenital defects. The inconstant and transient systolic murmur and thrill of congenital idiopathic dilatation of the pulmonary artery is a characteristic feature of that entity and is not a salient finding in any of the other anomalies listed above. The typical ''machinery murmur'' of patent ductus arteriosus (found in 95 per cent of the cases by Gross[9], and the similar systolic and diastolic murmurs of aortic-pulmonary septal defect are diagnostic of the respective anomalies. The rest of the congenital defects under consideration have no unique murmurs that are of aid in differential diagnosis.

The systemic blood pressure may be of value in differential diagnosis. The large pulse pressure, usually present in patent ductus arteriosus and aortic-pulmonary septal defect, and rarely in interventricular septal defect and atrial septal defect, is not found in patients with congenital idiopathic dilatation of the pulmonary artery.

The electrocardiogram may be of great help clinically, since a normal electrocardiogram, as is always found in congenital idiopathic dilatation of the pulmonary artery, is uncommon in the other anomalies. Right heart strain or right bundle branch system block is almost always found in isolated pulmonary stenosis. Similarly, right bundle branch system block is a very frequent finding in atrial septal defect, the incidence having been reported as high as

95 per cent.[10] Left heart strain is a consistent finding in aortic-pulmonary septal defect and a frequent occurrence in the late stage of patent ductus arteriosus; however, early in the course of the latter the electrocardiogram is usually normal. Interventricular septal defect may be accompanied by left or right heart strain, or the electrocardiogram may show abnormalities highly suggestive of congenital heart disease.[11]

In evaluating the x-ray and fluoroscopic examinations, it is necessary to compare the size of the pulmonary artery with the smaller peripheral pulmonary arteries. The vascularity of the peripheral lung fields should therefore be determined on a relative basis comparing it with the size of the pulmonary artery. In congenital idiopathic dilatation of the pulmonary artery there is normal vascularity, whereas isolated pulmonary stenosis is generally characterized by avascular peripheral markings. The rest of the congenital anomalies considered in the differential diagnosis generally show increased vascularity in the periphery of the lungs. If there is roentgenologic evidence of cardiac chamber enlargement, the diagnosis of congenital idiopathic dilatation of the pulmonary artery should be excluded. The other defects may be associated with normal heart size and chamber relationship. However, right ventricular enlargement is frequently noted in isolated pulmonary stenosis, while right ventricular and auricular enlargement is seen in atrial septal defect, and left ventricular enlargement is found in aortic-pulmonary septal defect. Less commonly left and/or right ventricular enlargement occurs in interventricular septal defects, and left ventricular, left auricular and/or right ventricular enlargement may develop as a late manifestation of patent ductus arteriosus.

It is to be emphasized that adynamic lesions with atypical clinical findings may be misleading. In these cases right heart catheterization is necessary to differentiate the various congenital anomalies described above.

Since relatively little is known of the natural history of idiopathic congenital pulmonary dilatation, a correct diagnosis serves a purpose more important than that of exclusion of other diseases. It will make it possible to have a long term follow-up on these patients, and in this manner to arrive at a proper prognosis of this condition. At the present time we feel that the dilated pulmonary artery probably represents only one aspect of a maldevelopment of the entire pulmonary tree as suggested for isolated pulmonary stenosis.[12] Although idiopathic dilatation of the pulmonary artery appears to be a benign condition, it must be admitted that only the future will definitely decide this question. This warning was found in one of the patients (L. P.) who presented mild pulmonary hypertension. It is possible that this patient had arrived at a more advanced stage of the condition than the others examined, and that eventually all of them will fall victim to a more malignant disease, primary pulmonary hypertension[13] or primary vascular sclerosis.[14]* On the basis of present knowledge and experience, the stigma of heart disease need not be attached to a person with idiopathic congenital dilatation of the pulmonary artery.

*It is also remotely possible that this patient has thromboembolic primary vascular sclerosis.[15]

SUMMARY

1. Six cases of idiopathic congenital dilatation of the pulmonary artery with clinical data and right heart catheterization studies are presented.

2. From the observations made, certain criteria have been enumerated which can establish the diagnosis clinically in most cases. However, confirmation by right heart catheterization is suggested in every case.

3. The importance of differentiating this entity from those cardiac defects correctable by surgery is stressed.

4. It is emphasized that, at the present, this condition is probably benign; however, it will take a long term follow-up to arrive at a prognosis for this anomaly with any degree of certainty.

CASE 1.—J. H., an 18-year-old white girl, following examination for respiratory infection was first told she had "heart trouble," nonrheumatic, at 6 years of age. At no time during her life did she experience rheumatic manifestations or evidence of congenital heart disease. Upon x-ray examination for entrance into college, the patient was found to have a large pulmonary artery segment. She was sent to us for investigation with the tentative diagnosis of patent ductus arteriosus.

Physical examination revealed palpable pulsations over the pulmonary area in the sitting position with a short systolic thrill in the second and third left intercostal spaces. A soft early Grade II systolic murmur in the third left intercostal space and a short diastolic blow in the second left intercostal space were present. These murmurs were not to-and-fro but were heard briefly in each phase of the heart cycle. A Grade I apical systolic murmur was also heard. In the supine position the murmurs were of greater intensity. There were good peripheral arterial pulsations. Cyanosis, clubbing, venous distention, pedal edema, and hepatosplenomegaly were absent. Blood pressure was 115/70 mm. Hg. Blood Kahn was negative, and the hemogram was normal. Fluoroscopic and chest x-ray examinations revealed normal vascular marking in the peripheral lung fields. Expansile pulsations ("hilar dance") were not present, but a very prominent pulmonary artery segment was seen (Figs. 1 and 2). There was no cardiac chamber enlargement. The electrocardiogram was within normal limits showing a semihorizontal "electrical" position. Right heart catheterization and respiratory studies were carried out and the results recorded in Tables I, II, and III.

CASE 2.—D. R., a 28-year-old white woman, was told she had a "heart murmur" at the age of 3 years. Growth and development were not retarded, and cyanosis was never observed. She had never had rheumatic fever, scarlet fever, tuberculosis, or syphilis. Following her second uneventful pregnancy and delivery, she was sent to us for evaluation of the heart murmur. Physical examination revealed a Grade II to Grade III systolic murmur in the second intercostal space to the left of the sternum. The intensity of the murmur was quite variable from day to day. A Grade I diastolic blow was also transiently present in the pulmonic area. P_2 was accentuated and greater than A_2. The femoral artery pulsations were good. The blood pressure was 120/70 mm. Hg. Blood Kahn was negative, and the hemogram was normal. Fluoroscopic and x-ray examinations of the chest revealed a hyperdynamic aorta and pulmonary artery, with prominence of the latter. There was no cardiac chamber enlargement, and the peripheral lung fields showed normal vascularity. The electrocardiogram was within normal limits showing a vertical "electrical" position. The patient underwent right heart catheterization and respiratory studies. The results are recorded in Tables I, II, and III.

CASE 3.—S. J., a 13-year-old girl, was found to have a "heart murmur" at 2 months of age. Her growth and development were normal, and cyanosis had never been observed. There was no history of rheumatic fever, scarlet fever, tuberculosis, or syphilis. Physical examination revealed a systolic murmur and thrill in the second and third left intercostal spaces, more intense in the sitting position. The blood pressure was as follows: right arm 112/50 mm. Hg; left arm 110/52 mm. Hg; right leg 118/62 mm. Hg; left leg 120/64 mm.

Hg. Fluoroscopic and x-ray examinations of the chest revealed a prominent dynamic pulmonary artery. The vascularity of the peripheral lung fields was normal. The electrocardiogram was within normal limits. The "electrical" position was vertical, and sinus arrhythmia was present. Right heart catheterization and respiratory studies were carried out and the results are recorded in Tables I, II, and III.

CASE 4.—D. F., a 14-year-old Negro boy, completely asymptomatic, was referred for study because of a "murmur" that had been present since 18 months of age. There was no history of cyanosis, retarded development, rheumatic fever, scarlet fever, tuberculosis, or syphilis. A systolic thrill was palpable in the left third intercostal space, and a Grade II to III systolic murmur was heard best in the same area. It was noted that both the murmur and thrill changed in intensity with the position of the patient, phase of respiration, time of day, and from day to day. P_2 was accentuated and greater than A_2. There was no cyanosis or clubbing. The femoral artery pulsations were good. The blood pressure was 120/80 mm. Hg. The hemogram was normal and blood Kahn negative. Fluoroscopic and x-ray examinations revealed the pulmonary artery to be enlarged in both the posteroanterior and right anterior oblique views (Figs. 3 and 4). The pulsations of the main pulmonary artery trunk and its branches were prominent, but definite expansile pulsations ("hilar dance") were not seen. The aorta was not decreased in size. There was no evidence of any cardiac chamber enlargement. The lung fields showed normal peripheral vascularity. The electrocardiogram was within normal limits, and showed a semihorizontal "electrical" position. Right heart catheterization and respiratory studies were carried out and the data are presented in Tables I, II and III.

CASE 5.—L. P., a 16-year-old white boy, was perfectly well until two months prior to admission. At that time he noticed easy fatigability and dyspnea with exertion, associated with an upper respiratory infection. He was hospitalized for the above complaints, and proceeded to develop a thrombophlebitis of the left leg, which responded to bed rest and penicillin. Following complete recovery from the above episode, the patient was sent to us for evaluation. Past history did not include rheumatic fever, scarlet fever, tuberculosis, or syphilis. Physical examination revealed a very well-developed and well-nourished boy in no distress. There was no evidence of cyanosis or clubbing. Lungs were clear to percussion and auscultation. There was no evidence of residual thrombophlebitis. A Grade II blowing systolic murmur was heard best over the pulmonic area, and an inconstant Grade I diastolic murmur was present also in the second intercostal space to the left of the sternum. The intensity of the murmurs varied from time to time, and on occasion a murmur could not be heard. P_2 was split and greater than A_2. The blood pressure was 130/80 mm. Hg. Blood Kahn was negative and hemogram normal. Fluoroscopic and x-ray examinations of the chest revealed a prominent pulsating hyperdynamic pulmonary artery with large branches. There were no expansile pulsations ("hilar dance") or chamber enlargement. The peripheral vascularity of the lung fields was normal. The electrocardiogram was within normal limits, showing a vertical "electrical" position. Right heart catheterization and respiratory studies were carried out and the results are summarized in Tables I, II, and III.

CASE 6.—J. D., a 21-year-old nurse, was found to have a "heart murmur" on routine physical examination in 1946. She experienced no symptoms until 1949 at which time she observed dyspnea with moderate exertion. The dyspnea was definitely worse during periods of excitement and anxiety. There was no history of scarlet fever, rheumatic fever, tuberculosis, or syphilis. Cyanosis had never been observed. One year prior to admission to this hospital, the patient experienced a period of emotional stress which was accompanied by weight loss. Following resolution of the emotional problem, her weight increased and general well-being improved. Physical examination revealed a well-nourished asthenic female with no evidence of cyanosis or clubbing. The femoral artery pulsations were good. The blood pressure was 110/80 mm. Hg. The heart was normal in size to percussion. P_2 was accentuated and greater than A_2. A systolic murmur, varying from Grade II to Grade III in intensity, was heard best over the third intercostal space to the left of the sternum. A transient systolic thrill

was also present in that area. The murmur was definitely more intense in the sitting position and in deep inspiration. Blood Kahn was negative, and the hemogram was normal. The electrocardiogram was within normal limits and showed a vertical "electrical" position. Fluoroscopic and x-ray examinations of the chest revealed a dynamic pulmonary artery with prominent major branches. The peripheral vascularity of the lungs was normal. There was no cardiac chamber enlargement. Right heart catheterization and respiratory studies were performed and the results are recorded in Tables I, II, and III.

We are indebted to the physicians of these patients for permission to study them, and to the members of the catheterization team for their assistance. We are grateful to Dr. L. N. Katz for his advice in preparing this report.

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TREATMENT AND PROPHYLAXIS OF EXPERIMENTAL RENAL HYPERTENSION WITH "RENIN"*

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THE pathogenesis of experimental renal hypertension is still unsettled. The three principal hypotheses are alteration in the renin-angiotonin (hypertensin) mechanism, decreased secretion of a renal blood pressure regulating hormone, and increased formation of pressor amines by the kidney. Additional evidence is urgently needed not only to clarify the pathophysiologic mechanisms involved in experimental renal hypertension but also because the many similarities between experimental renal hypertension and essential hypertension suggest the possibility of a partial common pathogenesis. As one approach to the problem, our research group some years ago reported the production of an antibody, antirenin, to heterologous renins with certain interspecies reactions including neutralization of the pressor effect of dog renin by antirenin to hog renin produced in the dog.[1, 2] We also reported reductions in the blood pressures of renal hypertensive dogs with repeated injections of crude hog renin and stated that "probably the mechanism of these reductions in blood pressure involves an immune (antihormone) response to the heterologous hog renin, inasmuch as dog renin and heat-inactivated hog renin were shown to be without effect on the blood pressure of other renal hypertensive dogs."[3] We now have excellent evidence that chronic renal hypertensive dogs can be treated successfully with crude or semi-purified hog renins, and that the development of experimental renal hypertension in dogs can be prevented by prophylaxis with crude or semi-purified hog renins. These therapeutic and prophylactic effects are highly correlated with the antirenin titers. Our previous inability to obtain such a correlation[4-6] was due to the interfering effect of hog renal medulla in certain of the crude hog renin preparations which we used, particularly crude hog renin obtained from whole kidney. This hog renal medulla principle manifests its

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antagonizing effect to a limited extent even in the minute amounts present in semipurified hog renin prepared from whole kidney. The evidence presented below for antirenin (or an antibody to a protein closely related to renin) as the mechanism of the therapeutic and prophylactic effects of crude and semipurified hog renins, which Goldblatt and co-workers[7] and Helmer and Shipley[8] have reported on, constitutes support for the affirmative of the long-standing controversy regarding the primary role of renin (or some other closely related protein) in the pathogenesis of experimental renal hypertension in the dog.

METHODS

Male and female mongrel dogs were made hypertensive by a standardized modification of the Goldblatt technique of renal artery constriction using the lumbar, retroperitoneal approach. The ages of the dogs were unknown, but most were probably 1 to 4 years old at the time of renal artery constriction. The setting of the clamp on the renal artery was fixed by a locknut and determined by a three to one ratio of the palpable pulse proximal and distal to the clamp. Normal caliber of the renal artery was maintained or restored by the application of 2 per cent procaine solution. The setting of the clamp was relatively constant for a given size of artery, weight of dog, and estimate of body fat. In most animals bilateral renal artery constriction was performed at a three-weeks' interval, although in some contralateral nephrectomy was performed. This technique in the hands of seven members of our research group has resulted in sustained hypertension in all dogs operated on so far. One dog in five dies of malignant hypertension within one to three weeks following the second operation. The hypertension of the surviving dogs persists for years until the death of the

TABLE I. HYPERTENSION IN UNTREATED CONTROL DOGS AFTER BILATERAL RENAL ARTERY CONSTRICTION

DOG NO.	SEX	MEAN FEMORAL ARTERIAL BLOOD PRESSURE (MM. HG)		INCREASE IN BLOOD PRESSURE (MM. HG)
		BEFORE RENAL ARTERY CONSTRICTION	AFTER BILATERAL RENAL ARTERY CONSTRICTION	
		AV. AND S. D.	AV. AND S. D.	
1	M	108 ± 7.4	137 ± 7.3	29
2	M	126 ± 4.4	156 ± 6.7	30
3	M	116 ± 4.5	151 ± 5.4	35
4	F	107 ± 10.0	142 ± 7.6	35
5	F	124 ± 4.5	160 ± 7.0	36
6	M	114 ± 3.9	150 ± 10.8	36
7	M	113 ± 6.0	150 ± 8.4	37
8	M	109 ± 6.3	146 ± 7.5	37
9	F	130 ± 5.8	167 ± 6.1	37
10	M	115 ± 5.4	153 ± 6.1	38
11	F	112 ± 4.8	154 ± 6.0	42
12	F	140 ± 8.0	184 ± 8.7	44
13	F	121 ± 3.5	165 ± 5.1	44
14	F	121 ± 5.5	166 ± 9.7	45
15	F	108 ± 3.5	153 ± 9.3	45
16	F	123 ± 4.0	171 ± 6.8	48
17	F	124 ± 5.1	179 ± 8.0	55
18	M	96 ± 4.8	153 ± 6.9	57
19	F	122 ± 3.8	182 ± 6.0	60
20	M	99 ± 5.0	173 ± 5.0	74
21	F	94 ± 5.6	160*	
22	F	125 ± 7.9	170*	
23	F	120 ± 3.9	170*	
24	M	120 ± 5.3	180*	
25	M	114 ± 6.7	184	

*Died with clinical and pathologic signs of malignant hypertension.

Mean increase in blood pressure afer bilateral renal artery constriction (excluding dogs which died) 43±10.9.

animal from old age, intercurrent infection, cancer, or rarely malignant hypertension with renal insufficiency, but never from myocardial insufficiency or cerebral accident. The chronic (four months or more) hypertensions so produced are illustrated in Table I which lists normotensions and hypertensions for a typical group of 25 dogs subjected to bilateral renal artery constriction. Each normotensive and hypertensive level is based on a minimum period of two to three months and 12 to 18 pressure readings. The chronic hypertensions of this group averaged 43 mm. Hg, with a range of 29 mm. Hg (1 dog) and 74 mm. Hg (1 dog), one-half of the dogs showing hypertensions of more than 40 mm. Hg. A preponderance of the latter was selected for treatment studies, so that, as detailed in Tables II to VIII, the hypertensions of the 104 treated hypertensive dogs averaged 48 mm. Hg with extremes of 24 mm. Hg (1 dog) and 88 mm. Hg (1 dog).

Mean blood pressure readings were obtained by femoral artery puncture one to two times per week, with occasional comparison by the Hamilton manometer technique. Studies of blood urea nitrogen, urinalyses, and determinations of body weight and clinical condition were made at monthly or bimonthly intervals, or more often when indicated. In certain experiments renal clearances, employing creatinine and sodium para-aminohippurate,* were performed.

Most of the crude renin was prepared essentially by the method of Grossman,[9] except that acetone was employed as a dehydrating agent, and much associated protein was removed by precipitation at a pH of 4.0. The crude renin solutions† were usually equivalent to 1 Gm. of fresh tissue per milliliter and commonly contained 2.0 to 2.5 D.U. (Goldblatt dog units) of renin per milliliter with a purity of 1 D.U. per milligram of nitrogen. The crude renin solutions contained approximately 1 per cent protein, 0.01 per cent of which was renin, and were preserved with 0.5 per cent phenol. The semipurified renins were prepared from crude renin by the method of Marshall and Wakerlin,[10] which is an adaptation of the well-known Cohn cold alcohol method of plasma protein fractionation. Even the semipurified renin solutions which usually contained 25 D.U. per milliliter and represented a renin purification of 50 to 140 times (average 70 D.U. per milligram of nitrogen), still contained at least 99 per cent nonrenin protein, since Haas and Goldblatt[11] recently reported an electrophoretically non-homogeneous renin of 5000 D.U. per mg. nitrogen purity.

Treatment and prophylaxis usually involved daily intramuscular injections of crude or semipurified renins or similarly prepared control tissue extracts. The customary dose for crude renin was 1.0 Gm. of fresh tissue equivalent per kilogram equal to 2.5 D.U. of renin if the extract was prepared from renal cortex and 2.0 D.U. if the extract was prepared from whole kidney. However, amounts as large as 10 Gm. of fresh tissue equivalent (in 1.0 ml.) containing 18 D.U. of renin were employed in a few experiments. The dose of semipurified renin was commonly 2.5 D.U. per kilogram, but amounts as large as 10 or more D.U. were employed in some experiments. In a few experiments, depot injections of semipurified renin in a slow absorption medium were studied. The chronic hypertensive dogs subjected to treatment had hypertensions of four to eighty-one months' duration, the majority between six and twenty-four months. The treatment period for the chronic hypertensive dogs varied from four to eight months or more. After a control period of at least two months, most of the normotensive dogs on prophylaxis experiments were injected for three months prior to constriction of the first renal artery, during the three weeks between, and for one week or one month subsequent to constriction of the second renal artery.

Serums were examined for antirenin before treatment, monthly during treatment, and monthly, bimonthly, or when indicated after treatment. Antirenin determinations on heparinized plasmas showed close agreement with values obtained on corresponding serums. Hemolysis was kept at a minimum, although occasional slight tinging of a serum or plasma was demonstrated not to influence the value obtained for the antirenin titer. The possibility that greater degrees of hemolysis may increase the antirenin titer is now being studied.

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†Crude hog renins were supplied in large amounts by Dr. O. Kamm of Parke-Davis & Co., Detroit, during 1942 to 1947 and by Doctors E. E. Hays and J. B. Lesh of Armour Laboratories, Chicago, Ill., during 1951 to 1952.

The technique commonly consisted in mixing the test serum with a semipurified renin solution containing 2.0 D.U. per milliliter and assaying by intravenous injection of the mixture into an etherized, nephrectomized dog of approximately 10 kilograms. Ordinarily the mixture was injected within five to ten minutes after its preparation, but a longer period of two to three hours at room temperature (or overnight in the refrigerator) did not significantly alter the assay finding. The presence of antirenin, of course, was demonstrated by partial or complete neutralization of the pressor effect of renin. The usual dose of renin was 0.5 or 1.0 D.U. (0.25 ml. or 0.5 ml.) depending upon the sensitivity of the assay animal. The amount of test serum varied with the antirenin titer. For example, the amount of serum giving complete neutralization of 1.0 D.U. of renin was obviously 0.1 ml. for a serum having an antirenin titer of 10 antirenin units per milliliter. The volume of the mixture was brought to 2.0 ml. by physiologic saline. Tachyphylaxis to renin frequently developed in the assay animals but was suitably controlled by the injection of a renin and normal or renal hypertensive dog serum control mixture before and after one or several injections of renin and antiserum mixtures. Antirenin titers were expressed in antirenin units (A.U.) per milliliter, one A.U. being the amount of antirenin which will completely neutralize the pressor effect of one D.U. of renin. Titers were usually determined against semipurified hog renin, but also against semipurified dog renin. In a few experiments the antirenin titer of a serum was determined by adding an increasing number of D.U. of renin from a concentrated semipurified renin solution to one milliliter of the test serum. The titers obtained in this way agreed well with those of the method commonly employed. The serums or plasmas tested for antirenin were not only controlled with serums from untreated normotensive and untreated hypertensive dogs, but also with serums from animals treated with control tissue extracts.

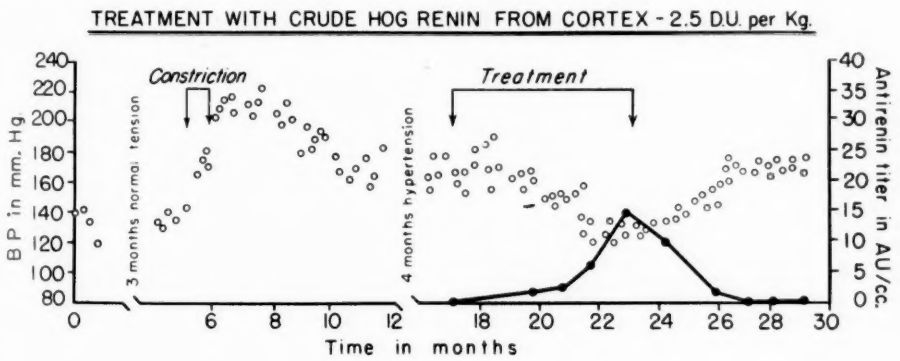
TREATMENT WITH CRUDE HOG RENIN FROM CORTEX - 2.5 D.U. per Kg.
Constriction
Treatment
BP in mm. Hg.
3 months normal tension
4 months hypertension
Antirenin titer in AU/cc.
Time in months

Fig. 1.

RESULTS

Our results are presented in two main categories; viz., (I) treatment of chronic experimental renal hypertension, and (II) prophylaxis of chronic experimental renal hypertension. Each of these is subdivided into results with (A) crude hog renin, (B) semipurified hog renin, and (C) control tissue extracts.

I. TREATMENT OF CHRONIC EXPERIMENTAL RENAL HYPERTENSION

(A) Crude Hog Renin.—(1) Table II shows the therapeutic effect of crude hog renin prepared from cortex free of medulla. In Table II, as well as Tables III to VIII, the normotensive and hypertensive levels are with few exceptions based on a minimum of 12 to 18 pressure readings over two to three months; the maximal response level is based on a minimum of 6 to 12 readings over

one to two months. The minimum effective antirenin titer for the dogs of Table II varied from 3 to 6 A.U. per milliliter of serum. The duration of the hypertension in these 12 dogs ranged from four to sixty-seven months, a majority being between seven and twenty-one months. The antihypertensive effects were well correlated with the antirenin titer. Fig. 1 shows an excellent therapeutic result (decrease in blood pressure to normotension). This 15 kg. male dog (No. 11) was treated for six months with daily intramuscular injections of crude hog renin from cortex in a dose of 1 ml. or 1 Gm. of fresh tissue equivalent containing 2.5 D.U. of renin per kilogram. The decrease in blood pressure was well correlated with the antirenin titer; as antirenin increased in titer during the fourth to the sixth months of treatment, the blood pressure fell to normotension even before the maximum antirenin titer was attained. Following the discontinuance of treatment, the blood pressure increased to the pretreatment hypertensive level as the antirenin titer decreased to 0. The other dogs in which a therapeutic response was obtained showed a similar inverse relationship between the antirenin titer and the blood pressure level.

TABLE II. ANTIHYPERTENSIVE EFFECT OF CRUDE HOG RENIN FROM CORTEX FREE OF MEDULLA

DOG NO.	SEX	DURATION OF HYPERTENSION (MONTHS)	MAXIMUM ANTIRENIN TITER (A.U./ML.)	BLOOD PRESSURE IN MM. HG			EFFECT ON HYPERTENSION IN PER CENT
				NORMOTENSION AV. AND S. D.	HYPERTENSION AV. AND S. D.	MAXIMUM RESPONSE TO TREATMENT AV. AND S. D.	
1	M	67	1	127 ± 10.3	167 ± 6.5	168 ± 5.5	+3
2	F	8		130 ± 8.2	178 ± 9.0	176 ± 8.2	–4
3	F	7	3	121 ± 13.7	152 ± 11.9	123 ± 8.2	–96
4	M	13		106 ± 5.8	137 ± 6.0	121 ± 6.5	–52
5	F	9	4	104 ± 12.3	167 ± 11.0	167 ± 9.9	0
6	F	37	6	128 ± 5.4	188 ± 8.1	148 ± 6.0	–67
7	F	4		125 ± 7.5	187 ± 5.3	156 ± 8.0	–50
8	F	4	10	110 ± 10.0	159 ± 12.1	121 ± 2.8	–78
9	F	4		127 ± 10.4	170 ± 7.7	129 ± 5.9	–95
10	F	18		109 ± 6.3	146 ± 7.5	110 ± 5.1	–97
11	M	21	14	128 ± 7.0	168 ± 10.0	128 ± 7.0	–100
12	F	4	90	105 ± 5.2	148 ± 3.9	110 ± 5.4	–88

Dog No. 12 in Table II with the antirenin titer of 90 A.U. has been maintained on treatment for twenty months to serve as an antiserum (antirenin) donor for passive immunization studies on other renal hypertensive dogs. The blood pressure of this animal has continued in the normotensive range. In the dogs of Table II, as well as the others of this report, antirenin titers were the same to hog and dog renins.

None of the dogs of this group showed adverse effects from treatment, except rarely local infection at an injection site. Appetite, body weight, and general clinical condition were well maintained. Urinalyses and blood urea nitrogen determinations consistently gave normal findings. The other chronic renal hypertensive dogs treated with crude and semipurified hog renins likewise showed no toxic or pyrogenic effects.

(2) Crude hog renin from cortex variably contaminated with medulla showed an over-all satisfactory therapeutic effect which varied with different preparations and questionably correlated with the antirenin titer. The thera-

peutic failures in the presence of good antirenin titers (Table III) we consider to be due to the interfering effect of hog renal medulla. The durations of the hypertensions in these 44 dogs varied from four to sixty-four months, a majority falling between six and twenty-four months. A decrease in blood pressure two-thirds toward normotension was obtained in dog No. 33 which had been hypertensive for sixty-four months.

TABLE III. ANTIHYPERTENSIVE EFFECT OF CRUDE HOG RENIN FROM CORTEX VARIABLY CONTAMINATED WITH MEDULLA

DOG NO.	SEX	DURATION OF HYPERTENSION (MONTHS)	MAXIMUM ANTIRENIN TITER (A.U./ML.)	BLOOD PRESSURE IN MM. HG: NORMOTENSION AV. AND S. D.	BLOOD PRESSURE IN MM. HG: HYPERTENSION AV. AND S. D.	BLOOD PRESSURE IN MM. HG: MAXIMUM RESPONSE TO TREATMENT AV. AND S. D.	EFFECT ON HYPERTENSION IN PER CENT
1	F	35	1	126 ± 4.5	175 ± 6.8	160 ± 3.0	−31
2	M	37	2	127 ± 10.2	168 ± 7.4	154 ± 5.2	−34
3	M	11		128 ± 5.3	181 ± 10.7	182 ± 7.1	+2
4	M	5	3	126 ± 4.1	170 ± 6.6	141 ± 7.4	−66
5	M	9		124 ± 2.4	169 ± 6.9	120 ± 7.9	−109
6	M	7		116 ± 5.7	185 ± 7.7	168 ± 11.5	−25
7	M	24		140 ± 17.2	212 ± 7.5	173 ± 5.0	−55
8	M	5		124 ± 3.6	170 ± 6.4	138 ± 7.4	−70
9	M	38		118 ± 4.0	168 ± 6.0	140 ± 7.1	−56
10	M	36		138 ± 10.5	200 ± 5.5	173 ± 6.8	−44
11	F	7	>3	132 ± 6.2	181 ± 6.4	157 ± 4.8	−49
12	M	6		138 ± 7.2	181 ± 9.0	148 ± 7.6	−77
13	M	14		120 ± 5.2	175 ± 6.1	143 ± 4.6	−58
14	M	24		112 ± 3.4	167 ± 7.1	109 ± 6.4	−105
15	M	31		125 ± 5.1	178 ± 7.4	141 ± 5.0	−70
16	M	17		126 ± 6.0	170 ± 8.8	126 ± 7.0	−100
17	M	15		132 ± 5.5	188 ± 8.5	134 ± 6.8	−96
18	M	15		116 ± 5.7	164 ± 7.5	127 ± 5.0	−77
19	F	4		125 ± 2.7	170 ± 7.7	137 ± 10.4	−73
20	M	4	4	114 ± 5.2	164 ± 8.9	115 ± 9.7	−98
21	M	5		126 ± 4.4	150 ± 6.7	130 ± 5.6	−83
22	M	13		123 ± 6.0	179 ± 6.3	167 ± 3.8	−14
23	M	19	5	111 ± 5.9	179 ± 3.1	151 ± 4.7	−40
24	M	15		121 ± 7.9	174 ± 7.3	142 ± 5.0	−60
25	F	8		126 ± 4.5	176 ± 8.6	173 ± 3.9	−6
26	F	22		85 ± 7.4	120 ± 4.0	115 ± 4.4	−14
27	M	5		140 ± 7.8	180 ± 9.4	164 ± 3.7	−40
28	M	21	10	116 ± 5.4	167 ± 6.3	140 ± 3.2	−53
29	M	24		114 ± 5.2	159 ± 8.1	120 ± 5.7	−87
30	F	22		110 ± 8.0	147 ± 9.8	139 ± 3.2	−22
31	M	26		125 ± 5.7	168 ± 10.2	126 ± 2.0	−98
32	M	8		124 ± 8.4	163 ± 6.5	149 ± 7.6	−36
33	M	64	15	124 ± 4.9	155 ± 6.6	133 ± 6.6	−71
34	M	12		138 ± 7.9	166 ± 3.5	138 ± 2.9	−100
35	M	6		124 ± 6.9	161 ± 5.7	159 ± 4.9	−5
36	F	6		123 ± 4.4	171 ± 9.9	151 ± 5.6	−42
37	M	7	20	122 ± 6.5	182 ± 7.0	148 ± 8.0	−57
38	M	5		99 ± 5.0	133 ± 8.4	123 ± 5.0	−29
39	M	22		110 ± 8.0	166 ± 9.3	148 ± 4.2	−32
40	M	14		133 ± 4.6	175 ± 7.7	159 ± 3.0	−38
41	M	7	25	113 ± 6.0	150 ± 8.4	141 ± 11.9	−24
42	F	36	50	115 ± 3.3	146 ± 7.0	146 ± 8.7	0
43	M	27	60	116 ± 10.0	167 ± 6.7	130 ± 7.6	−73

(3) With crude hog renin prepared from whole kidney there was a negligible therapeutic result indicating an antagonistic effect of hog renal medulla even in the presence of good antirenin titers (Table IV). The findings in these

20 dogs also serve as a control to demonstrate that the antihypertensive results obtained with crude hog renin from cortex are not due to nonspecific, foreign protein effects. The hypertensions varied from four to eighty-one months. The only therapeutic effects were seen in dogs No. 13 and No. 17 with hypertensions of four months. The crude hog renin used for these two dogs was prepared from kidneys obtained from hogs directly on the slaughter house floor, processed through a meat grinder, and immediately placed in acetone at –40° C., the defatting and dehydrating of the tissue being conducted at this temperature rather than at 4° C. as was true for the other crude hog renin preparations. Whether this variation in the method of preparation of the crude hog renin was in any way related to the therapeutic response is unknown.

Fig. 2 shows a finding typical for the group. This 14 kg. female dog (No. 20) was treated for six months with a concentrated crude hog renin solution from whole kidney in a per kilogram dose of 1 ml. or 10 Gm. of fresh tissue equivalent containing 18 D.U. of renin. In spite of an antirenin titer of 40 A.U. there was no antihypertensive effect.

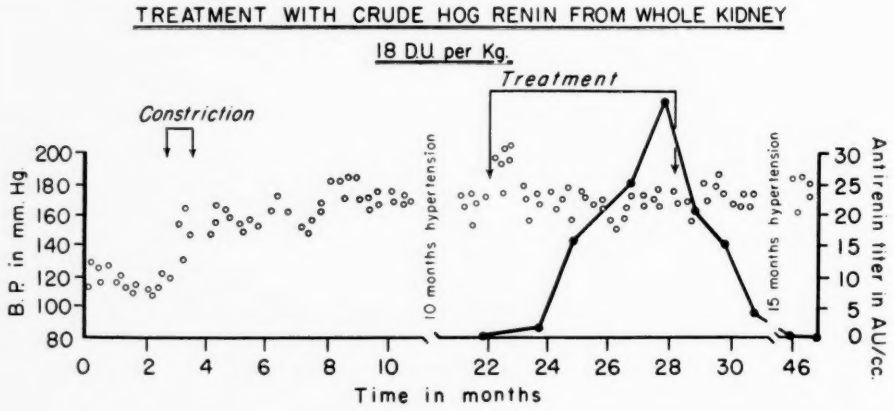

Fig. 2.

(B) Semipurified Hog Renin.—(1) Table V shows the excellent therapeutic effect of semipurified hog renin from cortex free of medulla. The duration of the hypertensions in these 9 dogs varied from eight to thirty-seven months. The minimum effective antirenin titre was 2 A.U. Fig. 3 shows an excellent therapeutic result in a 10 kg. male dog (No. 5) treated for six months with 2.5. D.U. per kilogram of semipurified hog renin from cortex (averaging 70 D.U. per milligram nitrogen). There was an inverse relationship between the blood pressure level and the antirenin titer.

Three of the dogs (No. 6, No. 8, and No. 9) of the group had previously received a five months' course of crude hog renal medulla extract prepared after the manner of crude renin in a dose of 1 ml. and 1 Gm. of fresh tissue equivalent per kilogram. On semipurified renin these dogs developed antirenin titers of 13, 15, and 20 A.U. and showed good, good, and excellent therapeutic responses, respectively. The previous course of crude medulla extract did not interfere with a satisfactory response to semipurified hog renin from cortex, perhaps because the latter was completely free of medulla protein.

(2) With semipurified hog renin from cortex slightly contaminated with medulla, there was a satisfactory antihypertensive effect (Table VI). The hypertensions of these dogs varied from eight to sixty months in duration. Good and excellent antihypertensive effects were obtained in two dogs (No. 4 and No. 6) which had been hypertensive for sixty and fifty-one months, respectively. Renal clearance studies were conducted on two of the dogs of this group (No. 1 and No. 5). Glomerular filtration rate (creatinine) and renal blood flow (para-aminohippurate) were determined before, during, and after treatment and showed no significant change, although moderate antihypertensive effects were obtained.

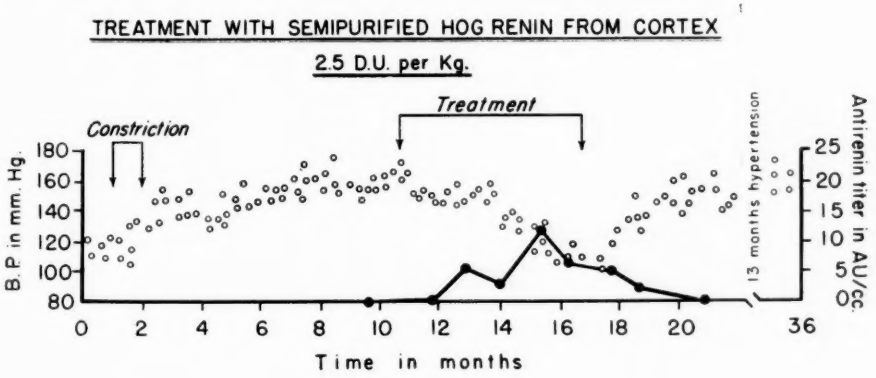

Fig. 3.

(3) Table VII suggests that the antagonistic effect of hog renal medulla protein operates through an immunologic mechanism which can be activated anamnestically. The semipurified hog renin slightly contaminated with medulla used for treating the dogs of Table VII was practically without therapeutic effect, even in the face of good antirenin titers, in the 6 chronic hypertensive dogs of Table VII which eight months previously had finished a course of crude hog renin from whole kidney. Three of the dogs in this group (No. 2, No. 4, and No. 5) were treated with semipurified renin in slow absorption media without any apparent advantage in regard to increased antirenin titer. Renal clearances in two dogs (No. 3 and No. 5) showed no significant change in glomerular filtration or renal blood flow during treatment.

(4) Treatment of 5 dogs with semipurified hog renin from whole kidney (in a purity averaging 70 D.U. per milligram nitrogen) showed a minimum effective antirenin titer of 11 A.U. (Table VIII). The hypertensions of the dogs of this group varied from five to fourteen months.

(C) Control Tissue Extracts.—Table IX indicates that no therapeutic effects were obtained with heat-inactivated crude hog renin from cortex and with heat-inactivated crude hog renin from whole kidney. This was likewise true for crude dog renin from cortex and from whole kidney. Obviously, heat-inactivated hog renins and the homologous dog renins did not give rise to antirenin. Crude hog renal medulla extract did not augment or otherwise alter

TABLE IV. ANTIHYPERTENSIVE EFFECT OF CRUDE HOG RENIN FROM WHOLE KIDNEY

DOG NO.	SEX	DURATION OF HYPERTENSION (MONTHS)	MAXIMUM ANTIRENIN TITER (A.U./ML.)	BLOOD PRESSURE IN MM. HG			EFFECT ON HYPERTENSION IN PER CENT
				NORMOTENSION AV. AND S. D.	HYPERTENSION AV. AND S. D.	MAXIMUM RESPONSE TO TREATMENT AV. AND S. D.	
1	M	45	0	110 ± 3.6	147 ± 5.7	153 ± 4.0	+16
2	M	27		125 ± 4.3	182 ± 5.0	181 ± 7.8	−2
3	M	27		129 ± 9.7	172 ± 7.9	178 ± 3.9	+14
4	M	5		114 ± 4.5	202 ± 5.3	223 ± 10.3	+24
5	F	8		108 ± 7.7	156 ± 5.5	163 ± 10.1	+15
6	M	81		115 ± 5.0	161 ± 5.4	160 ± 8.0	−2
7	M	35	2	139 ± 4.0	171 ± 4.6	174 ± 4.4	+6
8	M	5	3	122 ± 4.6	152 ± 5.3	151 ± 8.4	−3
9	M	4	5	110 ± 5.2	177 ± 9.7	170 ± 8.5	−10
10	M	18		120 ± 6.0	196 ± 8.9	190 ± 4.7	−8
11	M	13	6	102 ± 4.0	142 ± 7.7	143 ± 6.8	+3
12	M	21	10	112 ± 6.2	162 ± 8.1	154 ± 4.8	−16
13	F	4		111 ± 8.0	158 ± 6.0	132 ± 3.0	−54
14	F	9		86 ± 8.0	116 ± 4.3	111 ± 8.6	−17
15	M	26	12	133 ± 6.9	206 ± 11.3	209 ± 6.0	+4
16	F	23	15	122 ± 8.5	165 ± 7.7	160 ± 5.8	−12
17	M	4	16	111 ± 8.0	166 ± 11.0	139 ± 8.2	−49
18	M	10		124 ± 7.6	158 ± 8.5	154 ± 8.0	−12
19	F	40	25	115 ± 4.0	167 ± 6.4	172 ± 5.6	+10
20	F	13	40	114 ± 6.0	170 ± 8.6	165 ± 6.1	−10

TABLE V. ANTIHYPERTENSIVE EFFECT OF SEMIPURIFIED HOG RENIN FROM CORTEX FREE OF MEDULLA

DOG NO.	SEX	DURATION OF HYPERTENSION (MONTHS)	MAXIMUM ANTIRENIN TITER (A.U./ML.)	BLOOD PRESSURE IN MM. HG			EFFECT ON HYPERTENSION IN PER CENT
				NORMOTENSION AV. AND S. D.	HYPERTENSION AV. AND S. D.	MAXIMUM RESPONSE TO TREATMENT AV. AND S. D.	
1	M	17	2	114 ± 5.3	144 ± 5.0	122 ± 7.0	−73
2	F	12	6	130 ± 7.8	174 ± 7.0	128 ± 4.0	−104
3	M	8	7	123 ± 9.5	194 ± 11.0	153 ± 5.9	−58
4	F	17	8	113 ± 9.7	176 ± 6.9	127 ± 9.5	−78
5	M	14	13	115 ± 5.4	153 ± 6.1	115 ± 6.7	−100
6	M	19		93 ± 6.5	145 ± 7.8	108 ± 7.3	−71
7	F	26	14	110 ± 5.8	152 ± 7.2	112 ± 5.7	−95
8	M	16	15	96 ± 4.8	171 ± 7.9	124 ± 4.8	−63
9	M	16	20	114 ± 6.6	153 ± 6.9	115 ± 5.5	−97
10	M	37	48	116 ± 6.4	156 ± 7.1	117 ± 7.6	−98

TABLE VI. ANTIHYPERTENSIVE EFFECT OF SEMIPURIFIED HOG RENIN FROM CORTEX SLIGHTLY CONTAMINATED WITH MEDULLA

DOG NO.	SEX	DURATION OF HYPERTENSION (MONTHS)	MAXIMUM ANTIRENIN TITER (A.U./ML.)	BLOOD PRESSURE IN MM. HG			EFFECT ON HYPERTENSION IN PER CENT
				NORMOTENSION AV. AND S. D.	HYPERTENSION AV. AND S. D.	MAXIMUM RESPONSE TO TREATMENT AV. AND S. D.	
1	F	9	5	124 ± 5.1	179 ± 8.0	142 ± 5.1	−67
2	M	25	24	127 ± 10.1	179 ± 8.4	157 ± 6.5	−54
3	M	27		124 ± 4.5	161 ± 3.0	139 ± 5.2	−59
4	M	60		120 ± 6.8	187 ± 4.0	141 ± 11.5	−69
5	M	8	25	108 ± 7.4	165 ± 9.2	137 ± 7.3	−49
6	M	51	32	123 ± 4.9	166 ± 7.5	128 ± 8.9	−88
7	M	12	40	117 ± 10.0	172 ± 8.0	141 ± 3.0	−56
8	F	23	120	124 ± 3.7	170 ± 9.5	138 ± 4.5	−69

the hypertensive level. Other tissue extracts prepared after the manner of crude renin were without antirenin production and therapeutic effect. The treatment periods were the same as for crude and semipurified hog renins and the protein nitrogen injected with the control tissue extracts corresponded to that of crude hog renin.

TABLE VII. ANTIHYPERTENSIVE EFFECT OF SEMIPURIFIED HOG RENIN FROM CORTEX SLIGHTLY CONTAMINATED WITH MEDULLA EIGHT MONTHS FOLLOWING A COURSE OF CRUDE HOG RENIN FROM WHOLE KIDNEY

DOG NO.	SEX	DURATION OF HYPERTENSION (MONTHS)	MAXIMUM ANTIRENIN TITER (A.U./ML.)	BLOOD PRESSURE IN MM. HG: NORMOTENSION AV. AND S. D.	BLOOD PRESSURE IN MM. HG: HYPERTENSION AV. AND S. D.	BLOOD PRESSURE IN MM. HG: MAXIMUM RESPONSE TO TREATMENT AV. AND S. D.	EFFECT ON HYPERTENSION IN PER CENT
1	M	8	5	120 ± 7.8	170 ± 9.7	165 ± 8.3	−10
2	F	8	7	116 ± 8.5	146 ± 7.8	135 ± 3.5	−37
3	M	10	14	109 ± 4.6	163 ± 8.5	150 ± 5.3	−24
4	F	9	15	107 ± 5.6	141 ± 7.8	141 ± 5.3	0
5	M	10	20	107 ± 5.9	164 ± 8.0	154 ± 10.5	−18
6	M	9	80	108 ± 4.9	148 ± 3.8	147 ± 4.9	−3

TABLE VIII. ANTIHYPERTENSIVE EFFECT OF SEMIPURIFIED HOG RENIN FROM WHOLE KIDNEY

DOG NO.	SEX	DURATION OF HYPERTENSION (MONTHS)	MAXIMUM ANTIRENIN TITER (A.U./ML.)	BLOOD PRESSURE IN MM. HG: NORMOTENSION AV. AND S. D.	BLOOD PRESSURE IN MM. HG: HYPERTENSION AV. AND S. D.	BLOOD PRESSURE IN MM. HG: MAXIMUM RESPONSE TO TREATMENT AV. AND S. D.	EFFECT ON HYPERTENSION IN PER CENT
1	F	13	7	140 ± 8.0	184 ± 8.7	181 ± 8.6	−7
2	F	13		121 ± 5.5	166 ± 9.7	161 ± 5.1	−11
3	F	8	11	132 ± 7.1	171 ± 5.1	141 ± 4.7	−77
4	F	5	55	106 ± 8.7	148 ± 5.0	127 ± 5.0	−50
5	M	14	60	114 ± 3.9	150 ± 10.8	123 ± 3.0	−75

Of the 8 dogs injected with crude dog renin from cortex, 4 were given 2.5 D.U. of renin (1.0 Gm. of fresh tissue equivalent) per kilogram and 4 received three times this dose. Treatment was intramuscularly, daily, and for six months. Three dogs of the first group showed a maintained pressor effect averaging 30, 40, and 40 mm. Hg, respectively, and two dogs of the second group showed a maintained pressor effect averaging 20 and 40 mm. Hg, superimposed on their pretreatment hypertensions. In other words, the hypertensions of these five dogs gradually increased during the first two months of treatment, plateaued

TABLE IX. ANTIHYPERTENSIVE EFFECT OF CONTROL TISSUE EXTRACTS

EXTRACT	NO. OF DOGS	MAXIMUM ANTIRENIN TITER (A.U./ML.)	ANTIHYPERTENSIVE EFFECT
Inact. crude hog renin (cortex)	6	0	–
Inact. crude hog renin (whole kidney)	10	0	–
Crude dog renin (cortex)	8	0	–
Crude dog renin (whole kidney)	4	0	–
Crude hog renal medulla	4	0	–
Crude hog liver	3	0	–
Crude hog muscle	2	0	–
Hog plasma globulins	4	0	–
Crude beef heart	2	0	–

at the higher levels indicated during the next four months of treatment and then gradually decreased to the original hypertensive levels during the first two or three months following treatment. The remaining three dogs treated with crude dog renin from cortex showed no significant change in their hypertensive levels during treatment with crude homologous renin.

TABLE X. PROPHYLACTIC EFFECT OF CRUDE HOG RENIN FROM CORTEX FREE OF MEDULLA

DOG NO.	SEX	ANTIRENIN TITER AT TIME OF SECOND RENAL ARTERY CONSTRICTION (A.U./ML.)	BLOOD PRESSURE IN MM. HG			INTERPRETATION
			BEFORE TREATMENT	DURING TREATMENT		
				BEFORE CONSTRICTION	AFTER CONSTRICTION	
			AV. AND S. D.	AV. AND S. D.	AV. AND S. D.	
1	F	2	112 ± 5.1	117 ± 4.9	153 ± 7.1	*
2	F		130 ± 9.0	122 ± 11.2	120 ± 12.0	†
3	M	3	114 ± 6.8	120 ± 4.6	117 ± 9.0	†
4	F		114 ± 7.7	120 ± 4.5	121 ± 4.5	†
5	M	4	110 ± 6.5	117 ± 6.0	163 ± 6.4	*
6	F	5	132 ± 8.0	131 ± 6.7	132 ± 4.1	†
7	F	6	117 ± 8.0	118 ± 5.0	120‡	†
8	F	7	134 ± 6.6	131 ± 3.9	122 ± 7.3	†

*Not protected against hypertension.
†Completely protected against hypertension.
‡Died in renal insufficiency.

II. PROPHYLAXIS OF CHRONIC EXPERIMENTAL RENAL HYPERTENSION

(A) Crude Hog Renin.—(1) Table X shows the prophylactic effect of crude hog renin from cortex free of medulla. In Table X, as well as Tables XI to XV, the blood pressure level before treatment is usually based on a minimum of 12 readings over two months, the level during treatment and prior to renal artery constriction is similarly based, and the level after bilateral renal artery

TABLE XI. PROPHYLACTIC EFFECT OF CRUDE HOG RENIN FROM CORTEX VARIABLY CONTAMINATED WITH MEDULLA

DOG NO.	SEX	ANTIRENIN TITER AT TIME OF SECOND RENAL ARTERY CONSTRICTION (A.U./ML.)	BLOOD PRESSURE IN MM. HG.			INTERPRETATION
			BEFORE TREATMENT	DURING TREATMENT		
				BEFORE CONSTRICTION	AFTER CONSTRICTION	
			AV. AND S. D.	AV. AND S. D.	AV. AND S. D.	
1	F	1	130 ± 8.3	136 ± 7.0	178 ± 9.0	*
2	M	2	106 ± 4.7	117 ± 8.7	140‡	*
3	F		96 ± 5.0	110 ± 7.0	140 ± 3.0	*
4	M		90 ± 3.5	96 ± 5.5	126‡	*
5	F		100 ± 6.0	98 ± 8.1	105 ± 3.9	†
6	F	3	118 ± 5.8	115 ± 5.5	121‡	†
7	F		116 ± 4.9	114 ± 5.7	113 ± 7.0	†
8	M		114 ± 4.6	109 ± 5.9	106 ± 6.0	†
9	F	>3	115 ± 6.8	124 ± 5.2	144 ± 10.0	*
10	F		119 ± 7.0	116 ± 8.3	170 ± 9.0	*
11	F		127 ± 8.2	118 ± 6.7	118 ± 5.7	†
12	F		122 ± 3.0	122 ± 10.4	124 ± 10.5	†
13	F	5	115 ± 6.6	105 ± 3.0	96‡	†
14	M		96 ± 4.9	97 ± 5.7	100 ± 4.6	†
15	F	6	111 ± 5.0	125 ± 10.1	130 ± 6.7	†

*Not protected against hypertension.
†Completely protected against hypertension.
‡Died in renal insufficiency.

constriction is determined from a minimum of 6 to 12 readings over one to two months, except for the dogs dying in renal insufficiency four to eighteen days following the second renal artery constriction. The minimum effective antirenin titer for the dogs of Table X varied from 2 to 5 A.U. One of the dogs (No. 7) protected against experimental renal hypertension died in renal insufficiency four days after the second renal artery constriction. As stated under Methods above, one untreated control dog in five developed malignant hypertension with renal insufficiency, necrotizing arterial and arteriolar lesions, and death following our renal artery constriction technique. The nonsurviving dog (No. 7) of Table X was not protected against renal insufficiency and death but was protected against hypertension and against the necrotizing lesions.

Fig. 4 shows successful prophylaxis in an 11 kg. female dog (No. 8) with an antirenin titer of 7 A.U., resulting from injections of crude hog renin from cortex in a dose of 2.5 D.U. and 1 Gm. of fresh tissue equivalent per kilogram for three months prior to the first renal artery constriction, during the three weeks between constrictions, and for one week following the second renal artery constriction. When the antirenin titer decreased to 0 during the seventh month

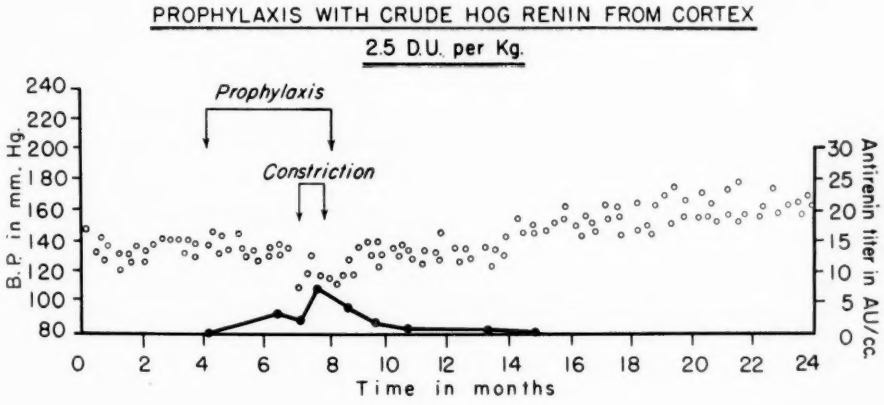

Fig. 4.

following prophylaxis, the blood pressure gradually increased to a definitely hypertensive level. The other dogs of Table X in which successful prophylaxis was achieved showed a similar inverse relationship between antirenin titer and blood pressure level following the discontinuance of renin injections, except that the increase in blood pressure usually occurred more promptly and less gradually as the antirenin titer fell. In general, this was true for all dogs protected against experimental renal hypertension by crude and semipurified hog renins as reported below. With the possible exception of certain dogs noted under II (A) (2) below, none of the dogs treated prophylactically with crude hog renin from cortex or with other crude hog renins showed any toxic effects.

(2) Table XI summarizes findings with crude hog renin from cortex variably contaminated with medulla. Except for 2 dogs (No. 9 and No. 10) which received a crude hog renin preparation which may have been heavily contam-

inated with medulla and which showed no protection in the face of an antirenin titer of more than 3 A.U., there was an excellent correlation of prophylactic effect and antirenin titer.

The prophylactic experiments on these fifteen dogs were conducted in three series of 4, 5, and 6 dogs, respectively, during successive years. No dogs died of renal insufficiency in the first series, one dog (No. 2) in the second, but 3 dogs (No. 4, No. 6, and No. 13) of the third series died of renal insufficiency, one protected and two not protected against experimental renal hypertension. The crude hog renin used for the third series of dogs was obtained by a long (forty-eight-hour) extraction of the defatted, dehydrated renal powder, instead of the usual sixteen to twenty hour period. The incidence of renal insufficiency and death for the third series, however, is not significantly different from the untreated control dogs or the dogs treated prophylactically with crude hog renin from cortex free of medulla ($P>0.05$).[12]

Glomerular filtration and renal blood flow in 6 of the dogs of Table XI showed no significant change prior and subsequent to the first renal artery constriction and showed approximately the same significant decrease in the 3 dogs (No. 3, No. 8, and No. 14) surviving the second renal artery constriction. Two of these dogs were protected against experimental renal hypertension and one was not.

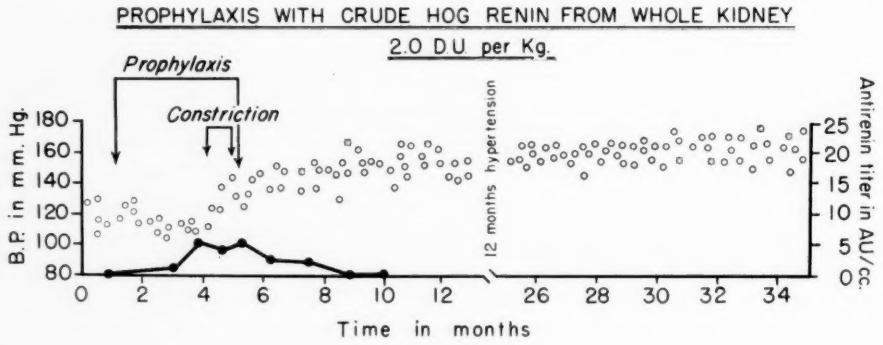

Fig. 5.

(3) The interfering effect of renal medulla on the prophylaxis of chronic experimental renal hypertension is shown in Table XII although failure of prophylaxis in a majority of the dogs is due to low antirenin titres (0 to 2 A.U.) Crude hog renin from whole kidney had a prophylactic effect in one dog (No. 16) with an antirenin titer of 5 A.U. Fig. 5 illustrates an unsuccessful prophylaxis in a 10 kg. female dog (No. 14) treated with crude hog renin from whole kidney in a dose of 2.0 D.U. (1 Gm. of fresh tissue equivalent) per kilogram and showing an antirenin titer of 4 A.U. at the second renal artery constriction. Three of the 20 dogs of Table XII died of renal insufficiency.

(B) Semipurified Hog Renin.—(1) Table XIII shows the excellent prophylactic effect of semipurified hog renin from cortex free of medulla. The minimum effective antirenin titer was 3 A.U.

TABLE XII. PROPHYLACTIC EFFECT OF CRUDE HOG RENIN FROM WHOLE KIDNEY

DOG NO.	SEX	ANTIRENIN TITER AT TIME OF SECOND RENAL ARTERY CONSTRICTION (A.U./ML.)	BLOOD PRESSURE IN MM. HG			INTERPRETATION
			BEFORE TREATMENT	DURING TREATMENT		
				BEFORE CONSTRICTION	AFTER CONSTRICTION	
			AV. AND S. D.	AV. AND S. D.	AV. AND S. D.	
1	F	0	116 ± 4.0	140 ± 2.0	173 ± 10.1	*
2	F		115 ± 9.5	132 ± 8.0	180‡	*
3	M		90 ± 3.0	91 ± 5.8	128 ± 8.6	*
4	M	1	135 ± 4.0	140 ± 8.1	165 ± 5.2	*
5	M		114 ± 6.5	110 ± 5.0	162 ± 8.0	*
6	M		115 ± 9.3	130 ± 8.1	182 ± 16.0	*
7	F	2	108 ± 6.0	117 ± 10.0	146 ± 5.5	*
8	F		116 ± 12.0	110 ± 4.0	136 ± 11.2	*
9	F		111 ± 8.6	106 ± 4.5	138 ± 4.8	*
10	M		113 ± 4.0	118 ± 4.0	149 ± 5.5	*
11	M		110 ± 7.0	105 ± 3.0	171 ± 11.1	*
12	M		112 ± 9.4	103 ± 4.5	186 ± 15.0	*
13	F	3	120 ± 7.9	116 ± 3.0	130 ± 4.0	*
14	F	4	95 ± 6.4	87 ± 6.4	120 ± 4.1	*
15	F	5	115 ± 7.0	110 ± 5.6	142 ± 9.6	*
16	M		123 ± 3.0	124 ± 6.5	125 ± 6.8	†
17	M		122 ± 6.0	116 ± 10.0	150‡	*
18	M		123 ± 5.0	122 ± 3.0	148‡	*
19	F	10	128 ± 4.9	132 ± 6.5	157 ± 11.0	*
20	M	22	110 ± 4.0	108 ± 4.9	130 ± 5.5	*

*Not protected against hypertension.
†Completely protected against hypertension.
‡Died in renal insufficiency.

Four of the 16 dogs of this group died of renal insufficiency and 5 others showed signs of uremia during the first seven to ten days following the second renal artery constriction. With one exception, these 9 dogs received a dose

TABLE XIII. PROPHYLACTIC EFFECT OF SEMIPURIFIED HOG RENIN FROM CORTEX FREE OF MEDULLA

DOG NO.	SEX	ANTIRENIN TITER AT TIME OF SECOND RENAL ARTERY CONSTRICTION (A.U./ML.)	BLOOD PRESSURE IN MM. HG			INTERPRETATION
			BEFORE TREATMENT	DURING TREATMENT		
				BEFORE CONSTRICTION	AFTER CONSTRICTION	
			AV. AND S. D.	AV. AND S. D.	AV. AND S. D.	
1	F	1	128 ± 12.0	126 ± 11.5	175 ± 7.1	*
2	F	3	117 ± 5.0	110 ± 7.9	115 ± 8.4	†
3	M		123 ± 3.7	120 ± 9.2	122‡	†
4	F	4	110 ± 5.9	111 ± 5.3	108 ± 7.5	†
5	M		136 ± 7.7	142 ± 5.6	138 ± 7.0	†
6	M		128 ± 6.1	128 ± 8.0	134 ± 4.0	†
7	F		137 ± 5.2	141 ± 8.7	136 ± 5.3	†
8	F	5	115 ± 4.5	121 ± 7.7	122 ± 5.6	†
9	F		108 ± 5.0	109 ± 9.2	105‡	†
10	F		130 ± 6.0	133 ± 6.8	127 ± 6.0	†
11	F	7	139 ± 6.4	135 ± 4.0	136 ± 5.0	†
12	F		127 ± 6.0	121 ± 3.3	122 ± 5.0	†
13	F	9	112 ± 4.3	118 ± 3.0	118 ± 4.1	†
14	M		138 ± 4.8	139 ± 5.2	134‡	†
15	M		123 ± 9.0	126 ± 3.5	120‡	†
16	M	12	125 ± 9.4	128 ± 8.1	126 ± 4.3	†

*Not protected against hypertension.
†Completely protected against hypertension.
‡Died in renal insufficiency.

TABLE XIV. PROPHYLACTIC EFFECT OF SEMIPURIFIED HOG RENIN FROM CORTEX SLIGHTLY CONTAMINATED WITH MEDULLA

DOG NO.	SEX	ANTIRENIN TITER AT TIME OF SECOND RENAL ARTERY CONSTRICTION (A.U./ML.)	BLOOD PRESSURE IN MM. HG			INTERPRETATION
			BEFORE TREATMENT	DURING TREATMENT		
				BEFORE CONSTRICTION	AFTER CONSTRICTION	
			AV. AND S. D.	AV. AND S. D.	AV. AND S. D.	
1	M	3	112 ± 5.1	113 ± 9.1	132‡	*
2	M	6	110 ± 6.8	111 ± 7.5	138‡	*
3	F		114 ± 3.0	124 ± 4.5	154‡	*
4	F	13	131 ± 12.1	126 ± 5.9	128‡	†
5	M	16	94 ± 7.6	98 ± 5.7	103 ± 6.9	†
6	F	20	117 ± 4.3	114 ± 6.0	117‡	†
7	F	22	126 ± 4.8	121 ± 3.5	118 ± 5.8	†
8	F	25	108 ± 5.0	104 ± 7.0	100‡	†
9	F	28	113 ± 8.0	106 ± 3.8	108‡	†
10	M	30	116 ± 6.5	109 ± 5.1	110 ± 5.0	†
11	M	80	97 ± 4.0	95 ± 3.0	84‡	†
12	M	120	105 ± 3.6	97 ± 5.8	90‡	†

*Not protected against hypertension.
†Completely protected against hypertension.
‡Died in renal insufficiency.

2.5 D.U. per kilogram of semipurified hog renin, whereas the other dogs were given 10 D.U. per kilogram. The possibility that the process of renin purification may remove a renal protective factor is suggested by the contrast between the morbidity and mortality of the dogs treated with the smaller dose of semipurified hog renin and those treated prophylactically with crude hog renin from cortex (Table X). The difference between the two groups is highly significant with $P = 0.001$.[12]

(2) Results with semipurified hog renin from cortex slightly contaminated with medulla are summarized in Table XIV. The prophylactic effect was excellent and highly correlated with the antirenin titer, although the minimum effective antirenin titer was 13 A.U. Nine of the 12 dogs in this group died in renal insufficiency, and the remaining 3 were uremic during the first two weeks after the second renal artery constriction. All 4 dogs given 2.5 D.U. of renin per kilo-

TABLE XV. PROPHYLACTIC EFFECT OF SEMIPURIFIED HOG RENIN FROM WHOLE KIDNEY

DOG NO.	SEX	ANTIRENIN TITER AT TIME OF SECOND RENAL ARTERY CONSTRICTION (A.U./ML.)	BLOOD PRESSURE IN MM. HG			INTERPRETATION
			BEFORE TREATMENT	DURING TREATMENT		
				BEFORE CONSTRICTION	AFTER CONSTRICTION	
			AV. AND S. D.	AV. AND S. D.	AV. AND S. D.	
1	M	6	108 ± 5.8	106 ± 4.6	140‡	*
2	M	10	138 ± 5.1	127± 5.4	160 ± 5.0	*
3	M		105 ± 8.5	104 ± 9.0	105 ± 3.0	†
4	M	13	116 ± 4.5	110 ± 9.8	142 ± 6.0	*
5	F	17	106 ± 6.0	100 ± 5.9	104 ± 4.0	†
6	F	34	130 ± 6.8	119 ± 5.0	127‡	†

*Not protected against hypertension.
†Completely protected against hypertension.
‡Died in renal insufficiency.

gram died; the other 8 received 10 D.U. per kilogram. The significantly high incidence of renal insufficiency and death in the 8 dogs receiving 10 D.U. of renin as compared with the 8 dogs [II (B) 1] receiving the same dose of semipurified hog renin from cortex free of medulla ($P = 0.001$), may be associated in part with the long extraction period (forty-eight to seventy-two hours) which was used in preparing the initial crude renin from which the semipurified renin given to the former group of dogs was obtained. We have already suggested that our renin purification process may remove a renal protective factor.

(3) Table XV shows results obtained in the prophylaxis of 6 dogs with semipurified hog renin from whole kidney. The minimum effective antirenin titer was 10 to 17 A.U.

(C) Control Tissue Extracts and Untreated Controls.—Table XVI indicates that no prophylactic effect was obtained with heat-inactivated crude hog renin from cortex or with heat-inactivated crude hog renin from whole kidney. This was likewise true for crude dog renin from cortex, as well as semipurified dog renin from cortex. Obviously, the heat-inactivated hog renins and the homologous dog renins did not give rise to antirenin. Crude hog renal medulla extract did not augment or otherwise alter the hypertensive response to renal artery constriction. Other tissue extracts prepared after the manner of crude renin were without prophylactic effect and antirenin production. That our method of renal artery constriction invariably produced hypertension is attested not only by the results in 32 dogs with control tissue extracts but also by the results in 56 untreated control dogs, all of which became hypertensive.

TABLE XVI. PROPHYLACTIC EFFECT OF CONTROL TISSUE EXTRACTS

EXTRACT	NO. OF DOGS	ANTIRENIN TITER AT TIME OF SECOND RENAL ARTERY CONSTRICTION (A.U./ML.)	PROPHYLACTIC EFFECT
Inact. Crude Hog Renin (Cort.)	4	0	–
Inact. Crude Hog Renin (Whole Kidney)	3	0	–
Crude Dog Renin (Cortex)	10*	0	–
Semipurified Dog Renin (Cortex)	2	0	–
Crude Hog Renal Medulla	4	0	–
Crude Hog Liver	3	0	–
Crude Dog Lung	2	0	–
Hog Plasma Globulins	4	0	–
None (untreated controls)	56†	–	–

*Three dogs of malignant hypertension.
†Eleven dogs died of malignant hypertension.

The three dogs dying of malignant hypertension in the group treated with crude dog renin from cortex belonged to a subgroup of 4 dogs given crude dog renin prepared by forty-eight-hour aqueous extraction, whereas the crude dog renin used for the other 6 dogs was prepared by the usual sixteen to twenty hour extraction. Again the results suggest that a long extraction period for defatted, dehydrated renal powder may give rise to a renotoxic principle in the extract, since the difference between these two groups has the borderline significance indicated by $P = 0.03$.[12]

DISCUSSION

The antihypertensive effects obtained in the treatment and prophylaxis of chronic experimental renal hypertension with crude and semipurified hog renins from cortex and semipurified hog renin from whole kidney are well correlated with the antirenin titers. As stated above, our crude and semipurified hog renins contained much nonrenin protein, inclusive of SPS (sustained pressor substance). Shorr and Zweifach,[13] however, were unable to demonstrate the presence of VEM in several of our preparations of crude hog renin and likewise found no anti-VEM in a limited number of our dog plasmas containing antirenin. With SPS, some of which was supplied by Shipley and Helmer, we demonstrated anti-SPS as well as antirenin in certain plasmas and in very limited studies found at least as good a correlation of the antihypertensive effect with anti-SPS as with antirenin. Obviously the antihypertensive effects which we report may be due to antirenin, to anti-SPS, or to an antibody produced by an as yet unidentified protein constituent of semipurified hog renin (or to two or more of these in combination).

The most cogent finding against the hypothesis that antirenin is responsible for the antihypertensive effects reported here is the interfering action of hog renal medulla. Thus the same plasma which assayed 40 A.U. per milliliter (Table IV) was without antihypertensive effect in chronic hypertension in vivo. The same was true for the prophylaxis dog with a titer of 22 A.U. per milliliter (Table XII). Obviously the antagonizing effect of hog renal medulla is not absolute since two hypertensive dogs in Table IV showed significant reductions in blood pressure with titers of 10 and 16 A.U. and one dog in Table XII showed successful prophylaxis with a titer of 5 A.U. per milliliter, these three dogs being treated with crude hog renin from whole kidney. The results with semipurified hog renin from whole kidney in treatment and prophylaxis demonstrated that the degree of semipurification employed does not remove all of the interfering renal medulla effect (Table XVII). Thus far we have not determined the mechanism of the antagonizing effect of hog renal medulla. Crude hog renal medulla extract was without effect in the treatment and prophylaxis of chronic experimental renal hypertension in the dog. The plasmas of dogs injected with medulla extract were without interfering effect on the neutralization of renin by antirenin either by the in vitro mixing of such a plasma with an antirenin antiserum and semipurified hog or dog renin and subsequent in vivo assay or by the direct intravenous administration of renin-antirenin mixtures to dogs

TABLE XVII. MINIMUM EFFECTIVE ANTIRENIN TITERS (A.U./ML. SERUM)

RENINS PREPARED FROM	TREATMENT WITH		PROPHYLAXIS WITH	
	CRUDE HOG RENIN	SEMIPURIFIED HOG RENIN	CRUDE HOG RENIN	SEMIPURIFIED HOG RENIN
Cortex	3-6	2	2-5	3
Cortex contaminated with medulla	3-60	5	2-5	13
Whole kidney	10->40	11	5->22	10-17

toward the end of a course of renal medulla extract. Moreover, the acute pressor effects of semipurified hog and dog renins intravenously appeared to be as well neutralized in dogs with antirenin produced by hog renins from whole kidney as from cortex. As part of our current study of the antihypertensive effect of passive immunization of renal hypertensive dogs with antirenin, we plan to compare the effects of equal quantities of antirenins to hog renin from whole kidney and to hog renin from cortex in the hope that such an experiment will elucidate the mechanism of the medulla antagonism. This comparison is particularly appropriate since the results of Table VII suggest that the medulla antagonism operates through an immunological mechanism.

There is, of course, the possibility that renin as the pathogenetic agent of experimental renal hypertension may not operate importantly through the hypertensinogen (renin substrate)-hypertensin (angiotonin) mechanism but through another route. Thus Sevy[14] of our laboratory recently reported that renin stimulates the anterior pituitary and that this action is blocked by antirenin. Certainly the effects of hog renal medulla and of hypothetical antibodies to renal medulla proteins on this renin-anterior pituitary mechanism should be studied. Another approach to the problem of renin as the pathogenetic agent via hypertensinogen-hypertensin involves the production of antihypertensinogen or of antihypertensin. Attempts in this direction have failed in our hands,[15, 16] probably because the amounts of hog hypertensinogen* used were too small and either because the amounts of hypertensin employed were too small or the latter molecule is not large enough to be antigenic. A conclusive experiment on the pathogenetic role of renin in experimental renal hypertension in the dog would be prophylaxis and therapy studies with a completely pure hog renin. As previously mentioned, the purest hog renin produced so far (5,000 D.U. per mg. nitrogen) is not electrophoretically homogeneous.

Recently Grollman[17] proposed that the antihypertensive effects of crude and semipurified hog renins were due to a nonspecific foreign protein response. This possibility appears to us to be completely eliminated by the numerous control tissue extract experiments cited, particularly those involving heat-inactivated crude hog renin from cortex. Indeed the absence of antihypertensive effects from these control tissue extracts is notable, especially since some of them were given in a daily dose of as much as 5 and 10 Gm. of fresh tissue equivalent per kilogram which meant approximately 50 and 100 mg. of protein per kilogram. Certainly the observation that bilateral nephrectomy in the peritoneally dialyzed dog leads to hypertension apparently through the loss of an incretory function of the kidney, in no way precludes the possibility that the kidney may under other circumstances originate pressor mechanisms leading to hypertension, even though Grollman appears to regard the former as completely excluding the latter.

The absence of toxic effects in our studies, except in certain prophylaxis experiments is noteworthy since Goodman and co-workers[18] and Masson and

*Crude hog hypertensinogen was supplied by Dr. E. E. Hays and Dr. J. B. Lesh of the Armour Laboratories, Chicago, Ill.

collaborators[19] have recently reported proteinuria and other adverse effects in the rat from the parenteral administration of hog renins of purity intermediate between the crude and semipurified renins which we have used. However, the kidney of the rat appears to be more susceptible to stressful and other adverse influences than the kidney of the dog. As previously stated, no adverse renal or other toxic effects were observed from crude or semipurified hog renins or control tissue extracts in any of the chronic renal hypertensive dogs. Likewise no adverse effects were observed in the prophylaxis dogs treated with crude hog renin or control tissue extracts obtained by sixteen to twenty hour extraction from renal or other tissue powders. The significantly higher incidence of death from renal insufficiency in the prophylaxis dogs treated with 10 D.U. of semipurified hog renin from cortex slightly contaminated with medulla and obtained from crude renin prepared by forty-eight to seventy-two hour extraction as compared with prophylaxis by semipurified hog renin from cortex prepared by sixteen to twenty hour extraction, suggests that a renotoxic principle is obtained by more prolonged extraction and not significantly removed by our process of semipurification of renin. Furthermore, the significantly higher incidence of illness and death from renal insufficiency in the prophylaxis dogs treated with 2.5 D.U. of semipurified hog renin compared with the prophylaxis dogs treated with crude hog renin both from cortex by short extraction suggests that a renal protective factor is removed by the process of semipurification of renin. Our method of renal artery constriction offers maximum opportunity for the demonstration of the presence of renotoxic and the absence of renal protective factors in the crude and semipurified renins studied, since the method produced hypertension in all untreated animals and malignant hypertension in 1 of 5. Obvious also is the fact that kidneys freshly subjected to renal artery constriction as in prophylaxis are more vulnerable to adverse influences than kidneys subjected to such constriction months or years previously as in the treatment of chronic experimental renal hypertension.

The chronic pressor effect observed in several hypertensive dogs with crude dog renin from cortex suggests but does not prove the possibility that the renal hypertensive dog may be more sensitive to or less well able to metabolize or destroy renin and/or one or more of the other vasoactive constituents of crude dog renin than is the normotensive dog. The possible importance of these observations to the pathogenesis of experimental renal hypertension is such as to warrant a detailed report which is in preparation.

In general, the prophylactic effects of crude and semipurified hog renins in chronic experimental renal hypertension in the dog are in complete agreement with the findings of Burns[20] of this laboratory in relation to the prophylaxis of malignant experimental renal hypertension in dogs with crude and semipurified hog renins from cortex and from whole kidney. Protection against the blood pressure increase was well correlated with the antirenin titer. Moreover, Burns' findings even more certainly suggest the presence of a principle or principles in the nonrenin fraction of hog renal cortex capable of protecting against the arteriolonecrotizing lesions, renal insufficiency, and death from malignant experimental renal hypertension.

Obviously trial of purified hog renin in essential hypertension in man is suggested by the findings reported. Unfortunately, however, antirenin to hog renin produced in man does not neutralize human renin and a course of semipurified hog renin in hypertensive humans has been shown to be without antihypertensive effect.[7, 8] Nevertheless, another approach is suggested by our finding that a passively produced antirenin titer is similarly effective antihypertensively to one actively produced in chronic renal hypertensive dogs.[21] Since we have shown that antirenin to human renin produced in the rabbit, dog and goat neutralizes human renin, we purpose to obtain sufficient quantities of purified antirenin to human renin from these species to study the effect of passive immunization of a group of patients with essential hypertension. If significant antihypertensive effects are obtained in any of the patients, a pathogenetic role for the kidney and the renin mechanism would be suggested. Should an appreciable percentage of patients with essential hypertension prove to have hypertension primarily or dominantly on a renal basis involving renin, various research groups would be stimulated to alter the antigenicity of hog renin or other animal renins to overcome the species difference which presently precludes the successful use of these renins in the treatment of human hypertension.

SUMMARY

Chronic experimental renal hypertension in dogs has been successfully treated with crude and semipurified hog renins and chronic experimental renal hypertension in dogs has been prevented by prophylaxis with crude and semipurified hog renins.

These therapeutic and prophylactic effects are well-correlated with the antirenin titer. This evidence for antirenin (or an antibody to a protein closely related to renin) as the mechanism of the antihypertensive effects of hog renins constitutes support for the affirmative of the long-standing controversy regarding the primary role of renin (or some closely related protein) in the pathogenesis of experimental renal hypertension.

Hog renal medulla contains a factor which interferes with the antihypertensive effects of hog renin in experimental renal hypertension in the dog by a mechanism presently unknown.

Renotoxic and renal protective factors have been found to occur in fractions of renal extracts. Toxicity consists in increased and protection in decreased incidence of uremia after constriction of the renal arteries.

After further preliminary experiments in animals, the possible pathogenetic role of the kidney in essential hypertension will be studied by passive immunization of patients with purified antirenin to human renin.

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GRAPHIC MEASUREMENT OF ARTERIAL PRESSURE IN THE UNANESTHETIZED RAT

An Improved Method

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DIRECT measurements of arterial pressure in rats require arterial cannulation under anesthesia, so that they have little value in the study of experimental hypertension. Indirect methods depend on detection of the resumption of arterial inflow distal to an occluding cuff on the tail[1-4] or the leg.[5-6]

Measurements on the tail have the theoretical disadvantage that they require that the animal be heated before and during the measurement, a process which increases arterial pressure.[7, 8] However, measurements on the leg or foot have the practical disadvantage that they require that the animal remain entirely still during the procedure and that the cuff be appropriately selected and carefully applied, so that these determinations are often tedious or sometimes impossible without anesthesia. An alternative is to select arbitrarily a rigid schedule of heating, so that measurements attain at least relative accuracy.[2-4] However, even with the microphonic end point of Friedman and Freed[4] and the photoelectric end point of Kirsten and associates,[5] the detection of arterial inflow is essentially subjective, so that investigators who accept measurements made in their own laboratories commonly suspect those made elsewhere.

We have therefore modified the device of Olmsted and associates,[6] which gives a graphic, objective record of the point of arterial inflow and adapted it to measurement of arterial pressure in the tail of the unanesthetized rat. The present report describes a comparison of this procedure with that of Friedman and Freed[4] and an evaluation of its accuracy as tested by simultaneous registration of carotid pressure at normal and hypertensive levels.

METHOD

I. *Apparatus.*—That for graphic recording is essentially that described by Olmsted and associates[6, 9] and the microphonic apparatus that of Friedman and Freed.[10]

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The graphic apparatus differs from that previously described in the application of cuff and strain gauge (Fig. 1) and in the driving mechanism for the pressure-recording pen (Fig. 2).

The cuff and strain gauge assembly are adapted to insertion and recording from the tail. The cuff consists of Penrose tubing (½ inch diameter, 1 inch long) mounted with twine in a brass tube (15 by 15 mm.), perforated by an air inlet tube which permits application of pressure by the cuff to the tail over an effective distance of 12 mm. The positioning of the strain gauge, inelastic pickup strap and Lucite tail holder are also shown in Fig. 1.

The cuff pressure is inscribed by a pen mounted in a Brush Penmotor which moves normal to the direction of paper travel. The pen-drive system is shown in Fig. 2. This apparatus is mounted on the right side of the recorder and at right angles to it. It consists of a 4-inch Fulton-Sylphon bellows, a double pulley system, and a strong radial spring.

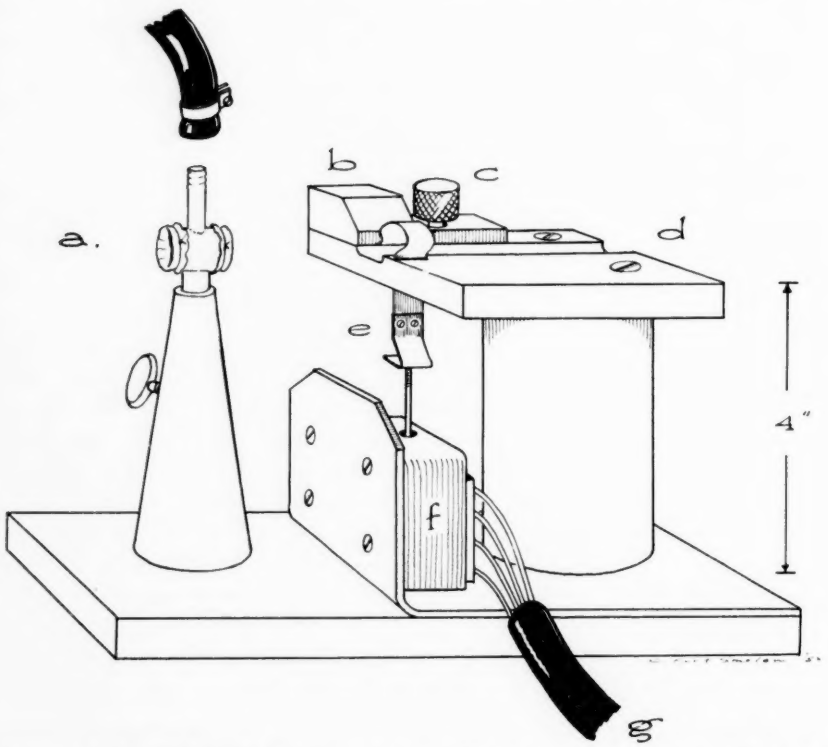

Fig. 1.—Cuff and strain gauge assembly. *a*, Tail cuff; *b*, clamping block; *c*, clamping screw; *d*, tail groove; *e*, strap; *f*, gauge; *g*, leads to amplifier.

Inflation of the bellows against an helical spring causes rotation of the concentric 1-inch pulley *b;* the pull of spring *c*—which is mounted on the 4-inch pulley *a*—permits slow rotation of pulley *a;* this rotation pulls the pen from the base line; the distance it travels is proportional to the pressure on the bellows, the mechanical amplification being 4 to 1. On deflation, return of the pen is compelled by the pull of a rubber band. The valve for inflation and slow deflation is that previously described[9]; a mercury manometer is inserted by a T-tube in the air line distal to the valve.

II. *Procedure.*—The rats are placed in a room warmed to 35° C. They are allowed to remain there for at least fifteen minutes, during which time the amplifier is turned on and

a calibration of pressure established. A rat is then placed in a warming box at 45° C. for exactly three minutes, after which it is transferred to a water-jacketed body holder which is maintained at 38° C.[2] The tail is then led through the cuff secured on the tail holder by firm application of the inelastic pickup strap. The recorder is then turned on and the adequacy of the pulse record verified. If a characteristic pulse record is obtained (Fig. 3), the cuff is rapidly inflated to a point greater than anticipated arterial pressure; adequacy of inflation is demonstrated by disappearance of pulse beat and its replacement by irregular waves due to tremor or regular slower waves caused by respiration. The cuff is then slowly deflated. The point of return of the regular pulse beat and the beginning of its conelike pattern indicate arterial pressure. This level is measured by reference to the calibration. Calibration is repeated at the end of each series of measurements.

Occasionally, particularly with untrained animals, the records are unsatisfactory, so that the measurements must be repeated after a delay of five or ten minutes. Sometimes an unsatisfactory record is remedied by adjustment of the pickup strap on the tail. The custom is to obtain and average two satisfactory records from each animal; these may usually be obtained within fifteen to thirty seconds of the beginning of the tracing. When the animals have been accustomed to the procedure, pressures can be measured in 20 during forty minutes.

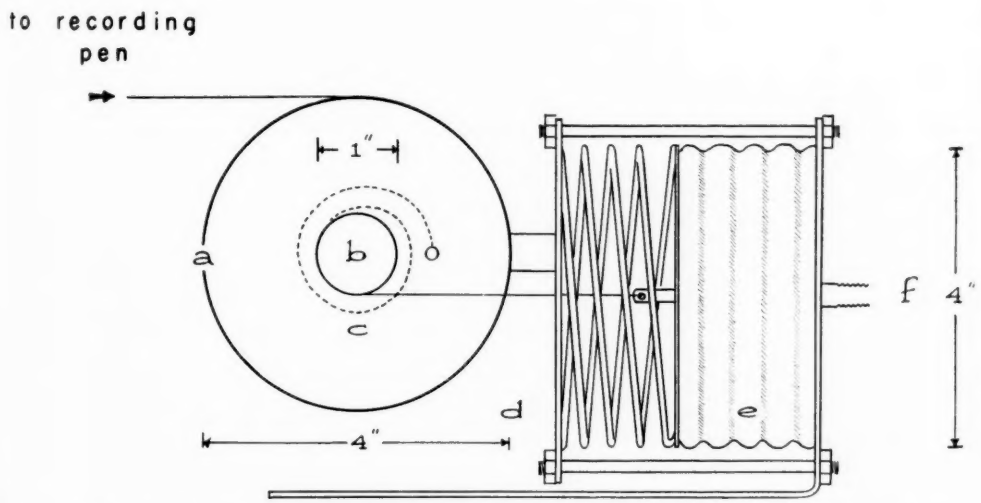

Fig. 2.—Driving mechanism for the pressure-recording pen. *a*, Four inch diameter pulley; *b*, one inch diameter pulley; *c*, weak spiral spring behind wheel opposed to helical spring; *d*, heavy helical spring; *e*, sylphon bellows; *f*, pressure inlet.

III. *Experimental.*—The comparisons of microphonic and graphic methods were done in groups of normal, unilaterally and bilaterally nephrectomized rats. The animals were of known strain, and weighed 150 to 250 grams. In normal and unilaterally nephrectomized groups, determinations were made alternatively by each method in the morning and evening on succeeding days; in the bilaterally nephrectomized, the measurements by each method were compiled sixteen to eighteen hours after removal of the second kidney, the operation having been done in two stages at four-day intervals.

The comparisons of graphic and carotid arterial pressures were done in 9 rats under light ether anesthesia. The registration of carotid pressure was from the record yielded by a Technitrol capacitance manometer[11]; the carotid artery was cannulated with a Petersen catheter; 44 records were obtained in untreated animals, and 37 at varying intervals in 6 rats after intravenous injection of 0.2 c.c. of a renal extract containing renin.

Fig. 3.—Tracings from normal (*I* and *II*) and hypertensive (*III*) rats. Direction of paper from right to left; arrows and numerals indicate blood pressure estimates.

RESULTS

I. *Comparison of Tail Methods.*—The data are summarized in Table I. They demonstrate that, under these conditions, neither unilateral nor bilateral nephrectomy significantly alters the means of pressure as recorded by either method, although bilateral nephrectomy causes an increased variability of pressure as graphically recorded. The microphonically indicated pressure is consistently and significantly less than that recorded graphically and, in normal and unilaterally nephrectomized rats, microphonic measurements are more widely variable.

II. *Comparisons of Graphic Tail and Carotid Pressure.*—In the pretreatment periods, mean carotid average pressure was 102.5 mm. Hg (range 86 to 128, S.D. ± 11); graphic tail mean was 96.4 mm. Hg (range 80 to 127, S.D. ± 15). The difference between the means is not significant.

The observations after renin injection are graphically recorded in Fig. 4, from which it can be seen that the measurements are not in strict concordance, and that as the carotid pressure rises to high levels, the graphically indicated pressure lags somewhat behind. The regression equation[12] of the association was calculated and found to be $C = 1.21\ T - 15$ mm. Hg where C is the average of carotid pressure and T is the indicated tail pressure.

TABLE I. RESULTS OF MEASUREMENTS BY GRAPHIC AND MICROPHONIC METHODS INDICATED ARTERIAL PRESSURE MM. HG

	MICROPHONIC					GRAPHIC					
GROUP	MEAN	S.D.	S.D.M.	n	N	MEAN	S.D.	S.D.M.	n	N	P
Normal	97	±20	±3.4	36	6	133	±7.2	±1.1	45	9	0.01
Unilateral nephrectomy	106	±27	±5.2	29	6	132	±16.2	±2.6	41	7	0.01
Bilateral nephrectomy	99	±21	±5.6	15	5	121	±23.4	±4.2	32	6	0.01

[a]Summary of results by the two methods. S.D. (standard deviation), S.D.M. (standard deviation of the mean) and standard error of difference are calculated by methods described by Bradford Hill,[12] and P values obtained by reference to Fisher's table of t. n = number of observation and N = number of animals observed.

III. *Effects of Drugs: Other Observations.*—Graphic records were made at intervals of one to two minutes continuously for one hour after subcutaneous injections of Adrenalin in oil (1 mg.) in 8 rats and in 6 given 10 micrograms of noradrenaline (Levophed, Winthrop) and without registration of carotid pressure and at longer intervals in some animals given renin subcutaneously. With Adrenalin in oil, and with noradrenaline, the indicated arterial pressures were widely variable; the ranges observed after Adrenalin were, at fifteen minutes, 125 to 158 mm. Hg, at twenty-five minutes, 150 to 230, at forty-five minutes, 120 to 195, and at 50 to 60 minutes, 108 to 135. With noradrenaline, maximum pressures at fifteen, thirty, and forty-five minutes were, respectively, 142, 168, and 170 mm. Hg; the pulse rate regularly decreased after injection of this drug, whereas it was not substantially altered by Adrenalin. Both drugs initially greatly decreased the amplitude of the recorded tail pulse. Subcutaneous injections of renin in normal animals caused sustained elevations of

pressure for periods up to six hours; the range and duration of the pressure increase varied with the size of the dose; pressor responses were usually associated with bradycardia.

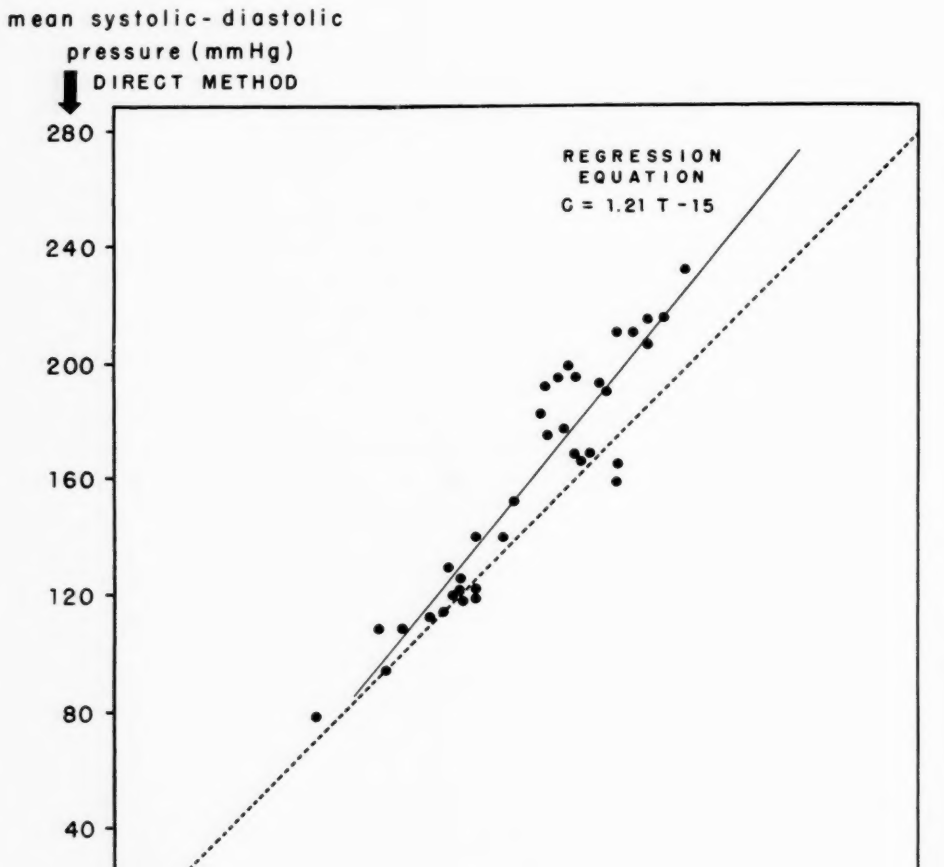

Fig. 4.—Association of simultaneous carotid average (systolic + diastolic/2) and indicated tail pressures in anesthetized rats.

The data presented in Fig. 4 as representing the correlation between tail and carotid pressures after intravenous renin are selected from measurements made two or more minutes after the injection. Initially, during the first rise of carotid pressure, tail pulse was greatly damped by this drug, and indicated pressures were erroneously low; this effect disappeared within a few minutes. Repeated injections of renin had diminishing and eventually no pressor effect.

DISCUSSION

Accuracy.—The accuracy of the graphic method for the registration of an approximation of mean arterial pressure at normotensive and hypertensive levels is shown by the data above. Indicated tail systolic pressure is less than carotid systolic pressure, presumably because the caudal artery is small. The association between indicated tail systolic and carotid mean pressure is close and, using the regression equation as a means of correcting for small disparities at hypertensive levels, the method yields a useful measure which approximates that obtained by carotid cannulation and a mercury manometer; the graphic registration can be made repetitively over long intervals in anesthetized animals without the risk of loss or difficulties inherent in carotid cannulation.

The observations with Adrenalin and noradrenaline suggest, and those made during the initial phase of action of intravenous renin show, that registration of caudal arterial pressure is not a valid means of estimating central arterial pressure during the persistence of a peripheral vasoconstriction with reduction of peripheral blood flow. The cycle of damping and recovery of the peripheral pulse after injection of renin corresponds roughly with the course of femoral arterial blood flow observed after injection of renin in the dog.[13] The skew of graphically indicated tail pressure from the line of perfect correlation with carotid pressure at hypertensive levels after renin may result from the persistence of a peripheral vasoconstriction five to ten minutes after injection, although such is not indicated by pulse amplitude.

Comparison with the Microphonic Method.—It has been demonstrated that the tail graphic method is inherently accurate. It follows that the wide difference in the means of indicated systolic pressure as between the graphic and microphonic methods is attributable to some systematic inaccuracy of the latter method which causes it to yield low estimates of the point of systolic inflow. This, however, is not a great disadvantage in a method which aims only to indicate relative arterial pressure, if the values indicated are sufficiently reproducible. However, the mean variability (coefficient of variation) of the graphic method in the three groups of rats compared is only 12 per cent, while that of the microphonic method is 22 per cent and, excluding the observations in bilaterally nephrectomized rats, these values are, respectively, 9 and 23 per cent.

This lesser variability of the graphic method indicates that it is relatively, as it seems to be absolutely, the more accurate method. It is presumably attributable in part to the objective nature of the end point. The microphonic method requires that the observer simultaneously recognizes an auditory perception, controls the pressure release valve, and watches his manometer, so that it places demands on his skill and training. The advantage of the permanent record yielded by the graphic method is obvious. This record also seems to aid in reducing the variability of the estimates. Thus, both methods are interfered with by movements of the rat's tail or body. However, with the graphic method, such artefacts are readily recognized, while, with the microphone, they often obscure or are even mistaken for the systolic signal. The

importance of rat movement as an artefact in the microphonic method is indicated by the narrow range of pressure observed by Friedman and Freed[4] in anesthetized rats, a demonstration which we have confirmed.

As noted above, duplicate graphic records of pressure can be secured from 20 rats—once they have been accustomed to the procedure—in thirty-five to forty minutes. Perhaps because of our lack of skill, a similar series of determinations by the microphonic method takes twice as long. However, Moss[14] was able to obtain 20 to 40 microphonic measurements per hour.

Thus, the graphic procedure has inherent advantages over the microphonic method, as it does over other presently available devices. Its greatest disadvantage is the cost of the apparatus and, in case of breakdown or electrical failure, its complexity.

SUMMARY AND CONCLUSIONS

1. A method is described for the measurement of arterial pressure in unanesthetized rats; the method involves graphic registration of tail pulse and pressure in a proximal occluding cuff.

2. The accuracy of the method was established by comparison of simultaneous tail graphic and carotid arterial pressures at normal, and at those hypertensive levels attained two or more minutes after intravenous injection of renin. The indicated tail systolic pressure correlates closely with the average carotid pressures.

3. A comparison of the tail graphic and microphonic methods was made; the microphonic method yielded substantially lower pressures, which were also two or three times more variable than those graphically recorded. Objectivity is the outstanding advantage of the graphic method; speed of operation is as great or greater than the microphonic; the cost of apparatus is considerably larger.

4. Observations after injections of renin, Adrenalin, and noradrenaline demonstrate that during the persistence of decreased tail pulse caused by any of these substances, tail pressures are either erroneously low in relation to central arterial pressure or immeasurable.

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VARIATIONS OF SERUM LIPIDS WITH AGE

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THE effect of aging on any function can be examined either by statistical analyses of data obtained from a large group of persons of all ages or by observations of the same individuals at different ages. Opportunities to collect data in man for considerable periods of time are rare. It therefore seemed worth while to repeat measurements of serum lipids on a group of subjects who were used to establish normal standards at earlier dates.

The serum lipids of 16 subjects, 7 males and 9 females, were remeasured after intervals of ten to twenty years. The only basis of selection was the availability of the subjects. Their ages varied from 20 to 48 years at the time of the initial observations, and from 30 to 65 years at the final observations. It was not possible to supplement the analyses of blood with other examinations to establish the "normality" of the subjects, but none of them had any known metabolic disorder and all were leading active lives. One, during the interval between observations, had undergone an operation for carcinoma of the rectum.

METHODS

Total cholesterol, lipid phosphorus, and total fatty acids were measured in the post-absorptive state before breakfast in every instance by identical techniques,[1] with one exception: for the recent determinations of lipid phosphorus the method had been adapted to a photoelectric instead of a visual colorimeter.[2] In the estimation of fatty acids of neutral fat it was assumed that 72 per cent of the total cholesterol was esterified.

RESULTS

The data are presented in Table II, the changes with age in Table III. Standards for the evaluation of the observed changes must be found. Studies of the variability of the lipids in the serum of normal individuals, measured by the same methods used in this study at intervals over fairly long periods, were published by Man and Gildea[3] in 1937. A portion of these data, somewhat expanded by additional measurements, is represented in condensed form in Table I. Six subjects are included, all of whom had at least six examinations over periods of three months or more. The seventh, S. H., is included with only three determinations because he happens to belong in the present series. Of the others M. G., E. M., and E. G. are also in the present series. E. M. and E. G. were studied most frequently and for the longest periods. The data from these two have, therefore, been subdivided into separate years. In all cases with more than three measurements the means and standard deviations have been derived by the method of least squares.

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TABLE I. VARIABILITY OF SERUM LIPIDS OF NORMAL ADULTS

SUBJECT (SEX)	AGE (YR.)	DURATION OF STUDY (MO.)	NO. OF OBSERVATIONS	CHOLESTEROL			FATTY ACID				LIPID P	
							TOTAL		NEUTRAL FAT			
				MAX.	MEAN	CORRECTED MEAN	MAX.	MEAN	MAX.	MEAN	MAX.	MEAN
				MIN. (MG. %)	S.D. (MG. %)	S.D. (MG. %)	MIN. (MEQ./L.)	S.D. (MEQ./L.)	MIN. (MEQ./L.)	S.D. (MEQ./L.)	MIN. (MG. %)	S.D. (MG. %)
B. A. (F)	25	3	6	265	248.7	254.4	14.7	13.74	3.6	3.07	10.8	10.41
				218	±15.9	±9.2	13.2	±0.60	2.5	±0.87	9.9	±0.36
M. G. (F)	32	12	9	182	169.3	169.3*	12.2	10.63	3.5	1.97	11.4	9.92
				154	±8.5	±8.5*	9.1	±0.98	0.1	±1.02	8.7	±0.71
E. M. (F)	30	8	12	252	224.8	222.2	12.7	11.77	2.5	1.69	11.3	10.45
				204	±11.0	±3.1	10.7	±0.61	0	±0.77	9.5	±0.57
	31	11	13	257	215.2	211.4	13.0	11.58	3.6	2.00	11.3	10.00
				203	±16.1	±7.2	10.0	±0.97	0.5	±1.07	9.3	±0.60
J. R. (M)	25	7	8	157	151.8	154.9	9.4	8.75	2.4	1.91	7.8	6.92
				128	±9.5	±3.7	7.8	±0.55	1.1	±0.37	6.4	±0.46
W. B. (M)	28	19	10	229	212.8	215.7	14.4	12.29	4.1	2.71	10.2	9.45
				185	±13.0	±9.9	9.9	±1.18	1.6	±0.71	8.4	±0.46
S. H. (M)	49	1	3	215	189.0		12.8	11.60	3.7	3.50	8.2	7.80
				160			10.5		3.1		7.6	
E. G. (M)	36	12	10	238	216.2	216.2*	13.4	12.53	3.4	2.71	11.2	10.05
				196	±11.7	±11.7*	11.5	±0.48	2.0	±0.37	8.7	±0.71
	37	13	12	239	211.9	211.9*	13.4	12.31	3.2	2.35	11.2	9.92
				173	±20.9	±20.9*	10.1	±1.03	1.6	±0.51	8.0	±0.92

*The means in these cases warranted no correction because the distribution of values was quite even (see text).

For cholesterol the maximum standard deviation is ±20.9 mg. per cent; for total fatty acid, ±1.18 meq. per liter; for the fatty acid of neutral fat, ±1.02 meq. per liter; and for lipid phosphorus, ±0.92 mg. per cent. When the figures for cholesterol were examined in detail, single measurements that diverged strikingly (more than 2 s.d. from the mean of all figures) from all others were found in all the subjects except M. G. and E. G. For example, the minimum value of 218 mg. per cent in B. A. is 28 mg. per cent below the next cholesterol, 246 mg. per cent, a value which is only 19 mg. per cent from the maximum, 265 mg. per cent. Similarly the maximum values, 252 and 257 mg. per cent in E. M., are completely out of line with all the other values in the thirty and thirty-one years' series. If such single aberrant values are omitted, the range of variation is greatly reduced. The corrected means with these figures omitted are shown in the third cholesterol column. In the case of M. G. the total variability was extremely limited; in the case of E. G., on the other hand, it was very great without any outstanding deviations. What these sporadic deviations in the other subjects may mean is not clear. They cannot be eliminated; but probabilities may be better expressed by the smaller ranges of variation in every subject but E. G. No similar deviations are found in any of the other lipid constituents. In all except the fatty acids of neutral fat the distributions are fairly even and symmetrical. The distribution of values for neutral fat fatty acids is quite capricious. This may be referable to biological reasons; it may be a mere mathematical consequence of the fact that they are estimated as a small difference between larger figures and therefore accumulate the errors of the other measurements.[1]

For purposes of comparison, the accepted limits of variation have been defined as twice the average standard deviation, including all values, not the corrected s.d.'s. These s.d.'s are the product of both biological and technical variations. The standards accepted are rigorous, yielding a theoretical expectancy of about 95 per cent. The accepted limit for cholesterol is ±30 mg. per cent (this excludes the 37 year s.d. of 20.9 of E. G.); for total fatty acid, ±1.6 meq. per liter; for fatty acids of neutral fat, ±1.5 meq. per liter; and for lipid phosphorus, ±1.2 mg. per cent. For reasons mentioned above, the limit for cholesterol may more nearly approximate ±20 mg. per cent.

There is no consistent trend in the cholesterol values of Table II. From Table III it may be seen that cholesterol rises six times and falls ten times. In two of the males, E. G. and S. H., it rises 61 and 57 mg. per cent, respectively; in M. M. it falls 57 mg. per cent. In three others, P. H., M. H., and P. L., it rises, while in D. H. it falls, slightly more than 30 mg. per cent. Although at least the first three of these probably represent significant changes, they cannot be related to aging per se, but must be attributed to some personal traits of the individuals. The curve of E. G. is peculiarly convincing because there is a progressive rise beginning between 37 and 41 years. This is the more convincing, as it will appear that all the lipid components follow a similar course.

The total fatty acids rise in all but four of the subjects, the increases equaling or exceeding 1.6 meq. per liter in nine. There is a significant decrease

TABLE II. SERUM LIPIDS AT DIFFERENT AGES

FEMALES						MALES					
			FATTY ACIDS						FATTY ACIDS		
SUBJECT	AGE (YR.)	CHOLESTEROL (MG. %)	TOTAL (MEQ./L.)	NEUTRAL FAT (MEQ./L.)	LIPID P (MG. %)	SUBJECT	AGE (YR.)	CHOLESTEROL (MG. %)	TOTAL (MEQ./L.)	NEUTRAL FAT (MEQ./L.)	LIPID P (MG. %)
M. G.	32	169*	10.6*	2.0*	9.9*	E. G.	36	216*	12.5*	2.7*	10.1*
	49	153	11.2	1.9	11.0		37	212*	12.3*	2.4*	9.9*
E. M.	28	215*	11.9*	2.3*	9.4*		38	220	14.3	5.0	9.0
	30	225*	11.8*	1.7*	10.5*		41	240	15.7		
	31	215*	11.6*	2.0*	10.0*		44	250	14.0		
	32	216*	13.1*	2.3*	10.4*		54	275	18.5	5.3	13.9
	38	203	11.2			S. H.	49	189*	11.6*	3.5*	7.8*
	47	183	10.6	1.4	10.0		65	246	16.8	6.0	10.7
P. H.	28	215	13.2	3.5	9.8	P. L.	26	199	12.2	3.6	8.4
	31	241	11.9	1.1	10.8		44	238	14.9	4.6	10.2
	47	248	15.4	4.6	10.6	J. P.	48	214	11.7	2.8	10.4
B. H.	20	180	10.7	2.2	8.8		65	198	14.1	5.1	9.1
	30	201	13.0	4.1	8.8	A. G.	29	246	13.4	3.2	9.6
E. S.	29	265	16.3	5.1	10.8		47	226	15.2	4.9	10.4
	46	237	12.8	2.5	10.2	D. H.	42	241	15.0	6.1	7.6
G. H.	32	214	12.0				59	202	14.9	4.5	11.4
	33	235	13.7			M. M.	44	230	14.2	4.0	10.1
	46	196	11.3	1.0	11.3		61	173	15.7	6.3	10.7
M. H.	35	219	11.6	1.0	10.0						
	53	254	16.3								
C. M.	45	250	13.1	1.8	11.4						
	62	234	14.1	2.4	12.5						
E. P.	42	195	13.1	4.0	9.4						
	59	192	17.4	7.5	10.8						

*Denotes more than one observation (see Table I).

TABLE III. CHANGES OF SERUM LIPIDS WITH AGE

SUBJECT	AGE INITIAL (YR.)	AGE FINAL (YR.)	CHOLESTEROL (MG. %)	FATTY ACIDS TOTAL (MEQ./L.)	FATTY ACIDS NEUTRAL FAT (MEQ./L.)	LIPID P (MG. %)
M. G.	32	49	−16	0.6	−0.1	1.1
E. M.	28	47	−32	−1.3	−0.6	−0.3
P. H.	28	47	33	2.2	1.1	0.8
B. H.	20	30	21	2.3	1.9	0
E. S.	29	46	−28	−3.5	−2.6	−0.6
G. H.	32	46	−29	−1.6		
M. H.	35	53	35	4.7		
C. M.	45	62	−16	1.0	0.6	1.1
E. P.	42	59	− 3	4.3	3.5	1.4
E. G.	36	54	61	6.1	2.7	4.4
S. H.	49	65	57	5.2	2.5	2.9
P. L.	26	44	39	2.7	1.0	1.8
J. P.	48	65	−16	2.4	2.3	−1.3
A. G.	29	47	−20	1.8	1.7	0.8
D. H.	42	59	−39	−0.1	−1.6	3.8
M. M.	44	61	−57	1.5	2.3	0.6
Average change*			−0.6	+1.7	+1.1	+1.0

*The sum of individual changes divided by the number of subjects.

in only one, E. S. In the group that rose are included all those with significant increases in cholesterol. The reductions of cholesterol were not associated with drops of fatty acid.

The fatty acids of neutral fat rose more than 1.5 meq. per liter in seven subjects, and fell more than 1.5 meq. per liter in two. These changes did not coincide in direction consistently with changes in either total fatty acids or lipid phosphorus.

Lipid phosphorus rose significantly, more than 1.2 mg. per cent, in five subjects. In two, E. G. and S. H., the increases were accompanied by rises of fatty acid and cholesterol; in the third, D. H., cholesterol fell 39 per cent while fatty acid did not change; in E. P., cholesterol did not change, while fatty acids and neutral fat rose.

DISCUSSION

On the whole, there is no evidence in the data that aging has a definite effect on the serum lipids. All of the components tend to rise significantly somewhat more frequently than they fall; but the difference in frequency is not great enough to warrant any inferences. It suggests rather that certain conditions which cause cholesterol to rise may preponderate over those that cause it to fall with advancing age. The apparently far greater frequency of rises in total fatty acids must be discounted, since this component represents the sum of changes in cholesterol esters, phospholipids and neutral fat, no one of which changed as consistently. In only two subjects, E. G. and S. H., did all components rise significantly. M. H. may possibly belong in the same class, but her lipid phosphorus was not measured. The data do not cover uniform intervals of age; but the directions of change do not appear to be distinctive at any particular intervals.

Keys and associates[4] measured serum cholesterol in males of different age groups above 30 years. They concluded that cholesterol rose gradually up to the age of 60 years, the rate of the increase between the ages of 17 and 45 years being 2.29 mg. per cent per year. Jones, Gofman, and co-workers[5] state that after the age of 30 years the serum cholesterol of both males and females increases slowly and steadily. Gertler, Garn, and Bland,[6] in a study of 146 healthy working men, found a steady and significant rise of serum cholesterol and phospholipids between the third and sixth decades. The rise of phospholipids was more clearly connected with age than was that of cholesterol.

Our data are more in keeping with those of Sperry and Webb,[7] who measured the serum cholesterol of fourteen men and eight women before and after intervals of thirteen to fifteen years. Cholesterol decreased in seven, the maximum fall being 33 mg. per cent, and rose 15 times by increments up to 53 mg. per cent. Sperry concluded that cholesterol may rise with age, but that this is not a consistent phenomenon. Kornerup,[8] from analyses of the sera of 125 adults and 96 children, concluded that in adults cholesterol and phospholipids are more closely related to the constitutional type than they are to the age of an individual. Page and associates,[9] in a study of sixty-five men, could detect no influence of age on serum cholesterol.

It might well be that if far larger series than Sperry and Webb's[7] or our own were studied in a similar manner, the dubious preponderance of increases of lipids observed in these series would become statistically significant for purposes of prediction. If, however, this significance depended not on a consistent trend but on the more frequent incidence of individuals with rising curves or the inclusion of a certain group with especially large increments, it would be improper to attribute the increases to age per se. In Sperry's series and our own, lipids sometimes diminish and sometimes remain constant as life advances. It must also be recognized that, if a biological function of this nature varies in both directions, increases may predominate mathematically over decreases for the simple reason that the floor is more resistant than the ceiling. This feature is evident in the systematic asymmetry (or skew) of the distribution of normal values for most constituents of blood.

SUMMARY

Total fatty acids, lipid phosphorus, and cholesterol of the serum of seven men and nine women were remeasured after intervals of ten to twenty years. The ages of the subjects at the time of the initial observations varied from 20 to 48 years, and at the final observations from 30 to 65 years. There was no consistency in the direction of change of any of the lipid components. Although they rose significantly more frequently than they fell, this tendency was not sufficiently preponderant to warrant the deduction that it is characteristic of the aging process.

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CEREBROSPINAL FLUID INORGANIC PHOSPHORUS IN NORMAL INDIVIDUALS AND IN THOSE WITH VIRAL INVOLVEMENT OF THE CENTRAL NERVOUS SYSTEM

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DATA on inorganic phosphorus in cerebrospinal fluids of normal individuals are very meager. This is understandable, since spinal punctures are not done routinely, but only when central nervous system involvement is suspected. Consequently, there are few, if any, completely normal values for inorganic phosphorus in the literature.

The levels of inorganic phosphorus in the cerebrospinal fluid in "normal" patients compiled from the literature are presented in Table I. However, only a small number of the patients can actually be termed "normal" because almost all were hospitalized, many of them for definite neurologic reasons: epilepsy, idiocy, cerebral agenesis, hydrocephalus, etc. We have excluded from Table I all values of inorganic phosphorus in the cerebrospinal fluid from patients with multiple sclerosis, syphilis of the central nervous system, meningitis, and encephalitis (demyelinating or destructive processes of the central nervous system), since we wished to compare the cerebrospinal fluid inorganic phosphorus levels in normal patients with those in the acute stage of viral involvement of the central nervous system.

It might be noted that Merritt and Fremont-Smith[10] pointed out that the phosphorus content of the cerebrospinal fluid is remarkably constant, but that it is increased in meningitic and other fluids with a high protein content. Furthermore, it is also interesting to advert to the determinations of enzymes in the cerebrospinal fluids by Demuth[11] and Kaplan, Cohn, Levinson, and Stern[12] which revealed that phosphatase was increased in pathologic conditions. Thus the increase in phosphatase would be expected to be accompanied by an increase in inorganic phosphorus.

The method used by different investigators (Table I) for the determination of inorganic phosphorus is basically the same: the formation of the heteropolyacid phosphomolybdic acid from orthophosphates (inorganic phosphates in the cerebrospinal fluid or serum) and molybdic acid and its subsequent reduction to the blue color "molybdenum blue." The choice of different reducing agents has produced the various modifications: Bell and Doisy[13] used hydroquinone, and Briggs[14] modified this by making the final solution acid; Tisdall[15] used potassium ferrocyanide and hydrochloric acid; Bodansky's[16] method employed stannous chloride originally described by Denigés; Fiske and SubbaRow[17] suggested 1,2,4-aminonaphtholsulfonic acid as the reducing agent; Gunther and Greenberg[18] determined calcium and phosphorus in the same sample by precipitating calcium first and then utilizing the supernatant filtrate for the analysis of inorganic phosphorus by the method of Fiske and SubbaRow.

The range of inorganic phosphorus from 170 determinations (Table I) was 0.8 to 2.6 mg. per 100 ml.; of these, 165 determinations were 2.0 mg. per cent or less, and only five were over 2.0 mg. per cent. The range of the averages of inorganic phosphorus found by different workers falls between 1.2 and 1.7 mg. per 100 ml.

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TABLE I. NORMAL VALUES OF CEREBROSPINAL FLUID INORGANIC PHOSPHORUS COMPILED FROM THE LITERATURE

AUTHORS	NUMBER PATIENTS OR FLUIDS	AGES	PATIENT STATUS OF "NORMALS"	RANGE INORGANIC PHOSPHORUS MG. PER 100 ML.	AVERAGE INORGANIC PHOSPHORUS MG. PER 100 ML.	METHOD
Cohen[1]	41	Children and adults	"No signs of organic nerve lesions"	1.25-1.9	1.64	Briggs[14]
Pincus and Kramer[2]	3	20, 28, 60 yr.	"Normal"	0.8 -1.8	1.23	Briggs[14]
Wilcox, Lyttle, and Hearn[3]	13	8 mo. to 11 yr.	Degenerative diseases of the central nervous system	1.3 -2.0	1.70	Tisdall[15]
Scholberg and Goodall[4]	51	16 to 56 yr.	Mental	1.17-2.24*	1.42	Bell and Doisy[13]
Steiner[5]	15	----	Not stated	1.4 -1.6	----	Bell and Doisy[13]
Lierle and Sage[6]	17	----	Asthma	1.2 -2.6†	1.65	Gunther and Greenberg[18]
Fremont-Smith et al.[7]	3	34, 42, 60 yr.	Pulmonary tuberculosis	1.2 -2.0	1.5	Fiske and SubbaRow[17]
Okuda[8]	14	2 to 3 mo.	"Healthy"	----	1.7	----
Cohn, Kaplan and Levinson[9]	13	----	Degenerative diseases of the central nervous system	1.0 -2.2‡	1.4	Bodansky[16]
Authors' data	22	9 mo. to 39 yr.	No apparent central nervous system involvement	0.9 -2.0	1.4	Modified Fiske and SubbaRow

*Fifty-one fluids from 36 patients; 49 fluids between 1.17 and 1.76 mg. per 100 ml.; the other 2 fluids were 1.93 and 2.24 mg. per 100 ml.

†Three values over 1.9; 2.3, 2.5 and 2.6 mg. per 100 ml.

‡Twelve values between 1.0 and 1.5; the thirteenth was 2.2 mg. per 100 ml.

There are many books, however, that give 3.5 mg. inorganic phosphorus per 100 ml. as the upper limit of normal for the cerebrospinal fluid.

These disparities in the normal values were perplexing; therefore, while evaluating the cerebrospinal fluid in measles encephalitis and encephalomyelitis (Odessky, Bedo, Jennings, Sands, Rosenblatt, Weisler, and Newman[19]) inorganic phosphorus determinations were done routinely on all patients on whom a spinal puncture was indicated.

MATERIAL AND METHODS

Patient Material.—This consisted of a group of 22 patients, 9 months to 39 years of age, admitted to the hospital from April through September, 1952, whose clinical picture on admission necessitated a lumbar puncture in order to rule out central nervous system involvement.

Methods.—The procedure employed to determine inorganic phosphorus in the cerebrospinal fluid was the modified Fiske and SubbaRow method which is commonly used for serum and can be found in many books on clinical methods, e.g.[20-24]

A résumé of the method with modifications for the smaller amounts of inorganic phosphorus in cerebrospinal fluid than in serum follows:

A protein-free filtrate of 2 ml. of cerebrospinal fluid was prepared by precipitating the cerebrospinal fluid proteins with 8 ml. of 10 per cent trichloroacetic acid, mixing and filtering through "ashless" grade paper. A 5 ml. aliquot of the filtrate was transferred to a cylinder or other container graduated at 10 ml. One milliliter of molybdic acid reagent and 0.4 ml. of aminonaphtholsulfonic acid reagent were added. Distilled water was added to the 10 ml. mark, and the solution was mixed. The blue color produced by the reduction of phosphomolybdic acid was allowed to develop for ten minutes and was then read in the Klett-Summerson photoelectric colorimeter with a red filter. The zero was set with a blank of 10 per cent trichloroacetic acid which had been carried through the same steps.

A stable stock standard phosphate solution was made up as for serum by dissolving 0.351 Gm. of pure dry monopotassium phosphate in water and transferring quantitatively to a 1 liter volumetric flask. Then 10 ml. of 10N sulfuric acid was added. Distilled water was added to the mark and the contents were mixed thoroughly. This solution contained 0.4 mg. of phosphorus in 5 ml. For cerebrospinal fluid analyses 5 ml. of this stock standard were transferred to a 100 ml. volumetric flask and made up to volume with 10 per cent trichloroacetic acid and mixed. A set of standards was prepared by taking three aliquots of 5 ml. each of the dilute standard solution, containing 0.02 mg. of phosphorus, and transferring to a cylinder or other container graduated at 20 ml. To the first cylinder, standard$_1$, 1 ml. of molybdic acid reagent and 0.4 ml. of aminonaphtholsulfonic acid reagent were added and distilled water was added to the 10 ml. mark. To the second cylinder, standard$_2$, 1.5 ml. of molybdic acid reagent and 0.6 ml. of aminonaphtholsulfonic acid reagent were added and distilled water was added to the 15 ml. mark. To the third cylinder, standard$_3$, 2 ml. of molybdic acid reagent and 0.8 ml. of aminonaphtholsulfonic acid reagent were added and distilled water was added to the 20 ml. mark. Each cylinder was mixed thoroughly, allowed to stand for ten minutes, and then read. Standard$_1$ contained 0.02 mg. phosphorus per 10 ml.; standard$_2$ contained 0.015 mg. phosphorus per 10 ml.; and standard$_3$ contained 0.01 mg. phosphorus per 10 milliliters.

Calculation:

$$\frac{\text{Density of Unknown}}{\text{Density of Standard}_1} \times 0.02 \times 100 = \text{mg. inorganic phosphorus per 100 ml. cerebrospinal fluid.}$$

For standard$_2$ 0.015 was substituted, and for standard$_3$ 0.01 was substituted in above.

Smaller Amounts of Cerebrospinal Fluid.—When, occasionally, a 2 ml. sample of cerebrospinal fluid was not available, to a 1 ml. (or 0.5 ml.) sample of cerebrospinal fluid was added 4 ml. (or 4.5 ml.) of 10 per cent trichloroacetic acid, and the solution was mixed and filtered. A 2.5 ml. aliquot was transferred to a cylinder graduated at 5 ml. and 0.5 ml. of molybdic

TABLE II. CEREBROSPINAL FLUID DATA OF PATIENTS WITHOUT CENTRAL NERVOUS SYSTEM INVOLVEMENT

CASE NO.	AGE YRS.	INORGANIC PHOSPHORUS MG. PER 100 ML.	CELLS PER CMM.	TOTAL PROTEIN MG. PER 100 ML.	GLOBULIN	SUGAR MG. PER 100 ML.	CULTURE	HOSPITAL STAY DAYS	CLINICAL SYMPTOMS ON ADMISSION	DISCHARGE DIAGNOSIS
1	¾	1.5	6	44	neg.	53	st.	8	History URI 1 wk.; rash 2 days; 104.4° F.; had generalized twitching and tremors in hospital; varicella eruption all over body	Varicella
2	11/12	1.6	0	39	neg.	65	st.	0	Observation poliomyelitis	Tonsillar abscess
3	1⅓	1.2	0	31	—	—	st.	4	Convulsions prior to admission; 104.0° F.; throat inflamed; Kopliks; cervical adenopathy; rash	Measles
4	1½	1.2	0	23	neg.	100	st.	6	Generalized convulsion 1 hr. prior to admission; 105.6° F.; some nuchal rigidity; pharynx injected; history exposure to varicella; 2 vesicles observed on abdomen	Tonsillitis
5	1½	0.9	58	32	v. ft. tr.	83	st.	6	History of recent head trauma; bloody tap; mild left hemiparesis	Subdural hematoma
6	1¾	1.7	0	26	neg.	82	st.	3	Convulsions pre-admission; 103.0° F.; cervical adenopathy; pharynx injected	Roseola infantum; acute pharyngitis
7	2	1.6	4	19	neg.	76	st.	6	104.6° F.; patient unable to stand today; multiple small glands at each side of neck and axillae; heavy nasal discharge; inflammation of eardrums, tonsils and pharynx; 1 hr. after admission patient had convulsion	URI; exanthum subitum
8	2½	1.0	1	18	neg.	82	st.	3	102.6° F.; mucous membranes, pharynx and tonsils inflamed; no Kopliks; moderate nuchal rigidity; history pain in left leg	Measles
9	3	1.5	0	15	neg.	92	st.	5	Observation poliomyelitis	Sickle cell anemia

10	3	1.4	0	19	neg.	95	st.	1	105.0° F.; convulsion before admission; mild rash	Pharyngitis
11	3½	1.6	0	34	neg.	83	st.	5	102.8° F.; mild nuchal rigidity; pos. Kernig; pos. Brudzinski; pharynx injected with small hemorrhagic spots on palate	Pharyngitis
12	4	1.2	0	13	neg.	82	st.	3	Observation poliomyelitis	Pharyngitis
13	5	2.0	0	19	neg.	70	st.	5	Convulsion prior to admission; 104.4° F.; drowsy; pharynx and tonsils inflamed and covered with white exudate	Tonsillitis
14	8	1.0	0	24	neg.	61	st.	7	103.0° F.; convulsion	Varicella
15	8	1.5	9	24	neg.	76	st.	2	103.0° F.; observation poliomyelitis	Acute pharyngitis
16	10	1.5	0	30	neg.	94	st.	3	Observation poliomyelitis	Acute pharyngitis
17	16	1.5	0	39	neg.	75	st.	0	Observation poliomyelitis	Pharyngitis
18	17	1.6	0	30	neg.	65	st.	0	Observation poliomyelitis; brother is a respirator case	URI
19	19	1.5	1	16	neg.	71	st.	8	Rheumatic heart disease for many years; 103.0° F.; complaining dizziness; severe pain in right leg and pain in neck "on and off"; depressed reflexes in lower extremity; pharynx and tonsils injected	URI
20	20	1.7	10	34	neg.	75	st.	4	Spastic tight hamstrings bilaterally; 101.0° F.; complains of ill-defined pains and aches; nausea; "stuffed" nose and pains in abdomen; is hungry	Pharyngitis
21	23	1.5	2	28	neg.	66	st.	4	Observation poliomyelitis	Hysteria
22	39	0.9	2	27	neg.	75	st.	2	Headaches; vomiting; stiff neck; knee jerk on right is absent; pharynx and tonsils inflamed; observation poliomyelitis	Influenza
Average		1.41								

acid reagent and 0.2 ml. of aminonaphtholsulfonic acid reagent were added. The solution was brought up to the 5 ml. mark with distilled water, mixed, allowed to stand for ten minutes, and then read. No change in calculation was necessary for the 1 ml. sample of cerebrospinal fluid since the final dilution to 5 ml. was equivalent to a 2 ml. sample diluted to 10 ml. For the 0.5 ml. sample the final result was multiplied by two.

RESULTS

Table II presents the data on the cerebrospinal fluids from patients without central nervous system involvement as well as the ages of these patients, their salient clinical symptoms, and final diagnoses. There were 22 patients whose

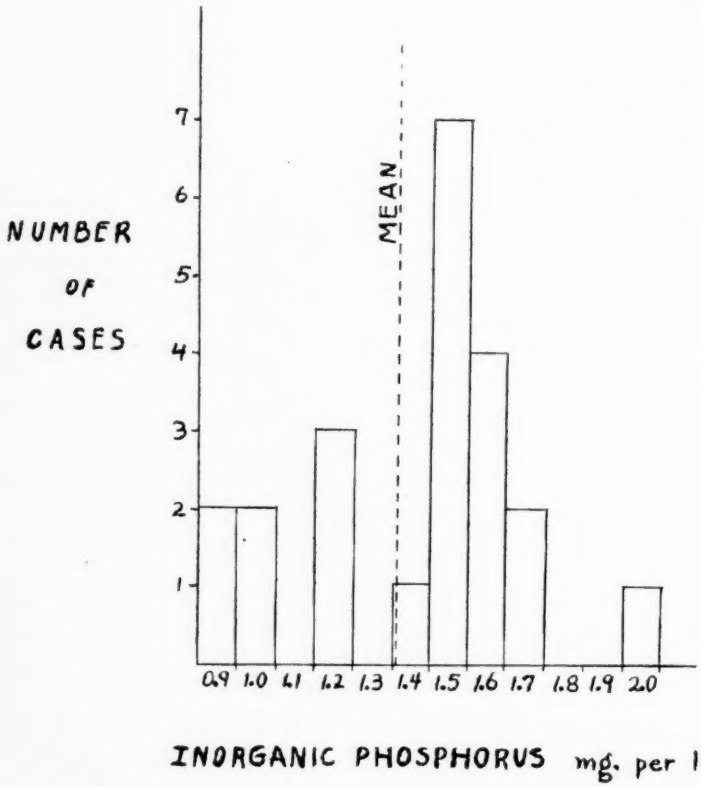

Fig. 1.—Frequency distribution of cerebrospinal fluid inorganic phosphorus of individuals thought not to have central nervous system involvement.

ages varied from 9 months to 39 years. All cerebrospinal fluids that showed pleocytosis, elevated total protein or traces of globulin were excluded with the exception of one fluid—Case 5—who manifested meningeal irritation from a subdural hematoma.

It was found that the average value of the inorganic phosphorus was 1.41 mg. per 100 ml. of cerebrospinal fluid (Fig. 1).

The mean value of 1.41 mg. per cent, the median and modal values of 1.5 mg. per cent and the fact that 21 out of 22 cases were 1.7 mg. per cent or

less indicate that the normal value for cerebrospinal fluid inorganic phosphorus is definitely less than 2.0 mg. per 100 ml. which is consistent with the findings of other investigators (Table I). The cerebrospinal fluid inorganic phosphorus levels of the 22 patients approximates a normal distribution, a slightly skewed frequency curve (Fig. 1). The standard error of the mean[25] was computed to be ± 0.06.

In contrast to the normal value of 1.41 ± 0.06 mg. per cent, the average value in the cerebrospinal fluid in the acute stage of measles encephalitis and encephalomyelitis[19] was found to be 2.02 ± 0.11 mg. per cent with a range of 1.3 to 4.1 mg. per cent (23 fluids from 12 patients) which represents the considerable increase of 43 per cent over our average normal value. The difference between the two averages, 1.41 and 2.02 mg. per cent, is 0.6 mg. per cent and is more than 3 times the sum of the standard errors $\left(\frac{0.6}{0.06 + 0.11} = 3.5\right)$ indicating that our data are extremely significant.

An increased level of inorganic phosphorus was similarly found in the cerebrospinal fluids of 105 patients during the acute stage of poliomyelitis (Odessky, Rosenblatt, Schapiro, Spielsinger, Riesenberg, and Landau[26]).

Increased values of cerebrospinal fluid inorganic phosphorus were also found in mumps meningoencephalitis, varicella encephalitis and influenza meningitis.[27]

DISCUSSION

Our results indicate that the normal value of inorganic phosphorus in the cerebrospinal fluid is less than 2.0 mg. per cent which is in agreement with the work of others. The finding of an increased cerebrospinal fluid inorganic phosphorus is significant diagnostically in viral infections.

Our 22 values from 22 patients have many inherent variables. At present we do not know whether cerebrospinal fluid inorganic phosphorus parallels that of the serum in that the serum inorganic phosphorus levels decrease with increasing age. Also, it could be justifiably argued that the average value, 1.4 mg. per cent, possibly may be slightly higher than normal because many patients were admitted with fever, a history of convulsions or exhibited neurologic disturbances (Table II).

In examining our data in viral involvement of the central nervous system it was observed that if the spinal puncture is done early in the acute phase, the pleocytosis appears to precede the increase in the total protein and inorganic phosphorus. Thus, pleocytic cerebrospinal fluids with little or no increase in total protein or inorganic phosphorus can be found. However, if the patient is acutely ill with a viral infection of the central nervous system, a definite increase in the total protein and inorganic phosphorus will be found at some stage of the disease process. As the infection subsides, the cell count will revert to normal first (less than 10 cells per cubic millimeter); the globulin may be negative or be present in only very faint traces; while the total protein and the inorganic phosphorus will remain elevated for some time longer, although the degree of rise in the total protein will not necessarily parallel that of the inorganic phosphorus.

The lack of parallelism in the increase of the total protein and the inorganic phosphorus may be due to the breakdown of varying amounts of nervous tissue—e.g., different amounts of proteins (globulins, nucleoproteins, nucleokeratin, etc.) and lipids (the phosphorylated phospholipids and the nonphosphorylated cerebrosides—an important constituent of medullated nerve fibers). These substances are not uniform in their protein and phosphorus content.

SUMMARY AND CONCLUSIONS

An average value of 1.4 mg. inorganic phosphorus per 100 ml. was found in the cerebrospinal fluids of 22 individuals who were thought not to have central nervous system involvement. The standard error of the mean was ±0.06. The range of inorganic phosphorus was 0.9 to 2.0 mg. per cent; 21 of the 22 values were between 0.9 and 1.7 mg. per cent.

The cerebrospinal fluid inorganic phosphorus is considered elevated if it is 2.0 mg., or more, per 100 ml.

The statistically significant increase in the level of inorganic phosphorus in the cerebrospinal fluid in virus diseases is pathognomonic of central nervous system involvement. Increased levels of cerebrospinal fluid inorganic phosphorus were found in measles encephalitis and encephalomyelitis, poliomyelitis, mumps meningoencephalitis, varicella encephalitis, and influenza meningitis.

Pleocytic cerebrospinal fluids with little or no increase in the inorganic phosphorus can be found in the early stage of acute viral infections of the central nervous system. As the infection subsides, the cell count will generally revert to normal first while the total protein and the inorganic phosphorus will take longer to return to normal values.

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BLOOD CEREBROSPINAL FLUID BARRIER IN MULTIPLE SCLEROSIS DURING ACTH ADMINISTRATION

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THE etiology and pathogenesis of multiple sclerosis remain obscure. The reported alteration in the neurologic findings of some patients with this disease during the administration of ACTH[1, 2] led to this investigation of the possible role of the blood cerebrospinal fluid barrier in the pathogenesis of multiple sclerosis on the premise that localized increase in capillary permeability of the vessels of the brain might allow the passage of a noxious substance producing scattered areas of demyelinization.

Such apparent increase in capillary permeability within the chronic plaques of multiple sclerosis has been demonstrated by Broman on autopsy specimens with the trypan blue technique.[3]

The suggestion that there is increased permeability of the blood CSF barrier in multiple sclerosis is possibly substantiated by the finding of a moderate increase in total CSF (cerebrospinal fluid) protein in 36 per cent of 203 patients with multiple sclerosis studied by Von Storch, Lawyer, and Harris.[4]

The effect of ACTH on vascular permeability of the vessels of the brain has been studied in cats by Prados, Strowger, and Feindel,[5] following exposure of the cerebral cortex to air. Their results indicate a marked reduction in vascular permeability following administration of pituitary corticotrophin and adrenal cortical extract as evidenced by inhibition of extravasation of perfused trypan blue into the brain substance and reduction in cerebral edema subsequent to exposure to air. Soffer and associates[6] interpreted the ACTH suppression of the Shwartzman phenomenon as due to a decrease in vascular permeability.

This study of the blood-CSF barrier, which has been localized to the epithelial cells of choroid plexuses and the endothelium of the cerebral vascular bed,[3] was done in human beings using the differential distribution of the bromide ion which is generally found to be three times as concentrated in the blood serum as in CSF[7] after oral ingestion. This test is probably more sensitive than measurements of the protein content of the CSF[3, 8] as evidence of an alteration in permeability of the blood-CSF barrier.

METHOD

Nine persons suffering from multiple sclerosis were placed on sodium bromide by mouth, 1.0 Gm. three times a day, for four-day intervals, before, during, and after a ten

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day administration of ACTH.* Blood and spinal fluid samples were taken on the morning after each four-day period to permit overnight equilibration of the last-ingested dose of bromide throughout the body. The levels of bromide in mg. per cent were determined colorimetrically within a known error of 5 per cent.[9] The ratio of the spinal fluid level to the serum level $\left(\frac{[Br^-]\ CSF}{[Br^-]\ S}\right) = R$ was taken as an index of the permeability of blood-brain barrier.

The treatment schedule for each patient is shown in Table I. Each patient was given 20 mg. of ACTH daily by slow intravenous drip over an eight-hour period for ten days. During the first five days of this period no bromide was given. On the sixth to the ninth days bromide was given, and on the tenth day the serum and spinal fluid levels were again determined. From one to three days after stopping both ACTH and bromide the third period of bromide administration was begun.

TABLE I. BROMIDE TREATMENT SCHEDULE IN RELATION TO ACTH ADMINISTRATION

DAY	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
NaBr 1 Gm. t.i.d.	x	x	x	x							x	x	x	x			x	x	x	x	
Blood CSF Bromide					x										x						x
ACTH 20 mg. IV/day						x	x	x	x	x	x	x	x	x	x						

Eosinophil counts were done before and after administration of ACTH on the first day, and in some cases were repeated on the seventh day. Serum levels of sodium, chloride, potassium, and carbon dioxide were determined before ACTH was given and repeated on the seventh day of ACTH administration. Spinal fluid protein levels were determined on all samples.

CLINICAL MATERIAL

Ten male veterans, varying in age from 23 to 34 years, and showing varying degrees of involvement with multiple sclerosis, were used in this series. Their histories ranged from two weeks to five years in duration of illness. Not all cases were admitted because of an exacerbation, and, frequently, statements concerning the onset and duration of symptoms were obscured by the patient's quest for secondary gain. In two cases there was urinary tract infection complicating the multiple sclerosis and in one of these there was also decubitus formation.

Summaries of four representative cases are presented (see Table II).

CASE 3.—This 29-year-old patient suffered progressive advance of his illness over a five-year period beginning with blurred vision and urinary hesitancy, then developing ataxia and subsequent spasticity. At the time of study he was bedridden, nearly blind, showed marked nystagmus, scanning speech, profound spasticity of all four extremities with clonus, and intention tremor. His course had been intermittently complicated by urinary tract infections and decubiti.

Although this patient had no objective clinical improvement, he showed changes in spinal fluid protein (reduction from 73.6 to 68.0 mg. per cent during ACTH and further reduction to 56 mg. per cent a week after it was stopped) and an upward trend in the bromide ratio from 0.56 to 0.77. This man retained bromide markedly, and high absolute serum and CSF levels developed that may have been a factor in the elevation of the ratio. He was the only patient to show bromide toxicity (drowsiness).

*ACTH supplied through courtesy of Armour & Co., Chicago, Ill.

TABLE II. SERUM AND CSF BROMIDE AND CSF PROTEIN DETERMINATIONS RELATED TO ACTH ADMINISTRATION

CASE NO.	BROMIDE LEVELS (MG. %)						RATIO $= \frac{[Br^-]_{CSF}}{[Br^-]_{S}}$			CSF PROTEIN LEVEL (MG. %)		
	SERUM (A)			CSF (B)								
	BEFORE	DURING	AFTER	BEFORE	DURING	AFTER	BEFORE	DURING	AFTER	BEFORE	DURING	AFTER
1	45.0	50.5	80.5	21.0	20.9	35.2	0.46	0.41	0.44	60	42	48
2	60.0	69.0	130.0	25.0	26.2	59.5	0.42	0.38	0.46	38	28	30
3	47.0	64.5	107.0	26.4	37.0	82.5	0.56	0.57	0.77	73.6	68	56
4	37.0	65.5	93.0	15.0	28.4	33.0	0.40	0.43	0.36	44	31	30.4
5	33.0	53.0	89.0	16.0	23.9	33.2	0.48	0.45	0.37	28	25	18
6	48.7	67.0	105.0	18.1	23.2	49.0	0.37	0.35	0.49	37	30	41
7	42.8	73.0	234	17.2	22.0	93.0	0.40	0.30	0.40	130	32	24
8	73.0	96.0	82.4	—	40.8	32.8	—	0.42	0.40	22.7	25	14.0
9	48.0	73.8	94.5	20.8	28.8	38.4	0.43	0.39	0.40	44	27	33
10	200.0			33.2			0.60			31		
DIAGNOSIS	*Nonmultiple Sclerotic Patients*											
Hysteria	33			12			0.36					
Hysteria	36			12			0.33					
Parkinsonism	55			20			0.36					
Amyotrophic lat. sclerosis	43.5	63.7	85	16	26.5	37	0.36	0.42	0.43	32.8	20	33
Syringomyelia	45.5			18			0.40					

CASE 5.—A 28-year-old married bus driver was well until one day prior to hospital entry when he experienced headache, diplopia, nausea, and vomiting. Physical examination revealed minimal ataxia, a mild intention tremor of the left arm and hand, and a left facial paresis. The most striking finding was a profound internuclear ophthalmoplegia. Laboratory findings revealed a normal spinal fluid and leukocytosis of 17,000 white blood cells per cubic millimeter. During the period of study and preliminary bromide testing (two weeks) the patient had a spontaneous and almost complete remission. He was given ACTH experimentally and showed no further clinical change. The spinal fluid protein dropped 10 mg. per cent and the ratio of CSF to serum bromide dropped from 0.48 to 0.37 between the "before" and "after" ACTH treatment studies. This patient returned after three months, complaining of numbness in the fingertips, but no objective changes were observed. There were no residua of the old findings.

CASE 8.—A 29-year-old telephone lineman noted numbness in the hands of four months' duration before entry, and subsequently developed difficulty in walking and starting the urinary stream. He demonstrated mild ataxia, mild spasticity of the lower extremities, Babinski signs bilaterally, slight hyperactivity of the knee and ankle reflexes, and diminished vibratory sensibility in the legs and feet. Routine laboratory studies of blood, spinal fluid, and urine were within normal limits.

There were no significant objective changes in this subject's clinical findings during or after ACTH. The bromide ratio was not appreciably altered between the ACTH and follow-up studies.

CASE 10.—This case was incomplete because of the sudden death of the 28-year-old patient in a convulsion following a heavy alcoholic debauch. He had, about four months before, developed an acute and massive left hemiparesis and showed extremity ataxia on the right side as well as spasticity on the left. Autopsy showed demyelinated plaques throughout the cord, particularly in the thoracic region. The patient's death occurred before ACTH administration, but the initial bromide ratio was high (0.60) and the case is included in the nonexperimental material for that reason.

In addition to the multiple sclerotic patients, the bromide ratio was determined on seven other patients, two of whom were considered to have hysterical reactions (ratios 0.36 and 0.33), a third suffering from Parkinsonism (ratio 0.36), a fourth with amyotrophic lateral sclerosis (ratio 0.36), and the fifth had syringomyelia (ratio 0.40). An asthmatic and an arthritic on ACTH also had single bromide ratio determinations, the former being 0.45 and the latter 0.36.

RESULTS

The ratios of spinal fluid bromide concentrations to serum bromide concentrations appeared slightly but not significantly higher than those of non-multiple sclerotic patients. One patient (Case 3), in whom a higher ratio was found, was febrile in association with a urinary tract infection. The change in the ratio determined at the time of ACTH administration was up (between 0.01 and 0.03) in two cases, down in six cases (between 0.02 and 0.10) and not determined in one (Case 8). None of these changes appeared to be of striking significance except possibly the decrease of 0.10 (Case 7). The ratios determined a week after cessation of ACTH administration showed a slight upward trend in seven cases (ranging from 0.01 to 0.20) of which three were slightly higher than the ratios found before ACTH was given. In all cases there was a definite increase in the absolute levels of bromide in the serum and spinal fluid—ranging from 5 to 35 mg. per cent in the serum between the first and second periods of bromide administration and 9 to 191 mg. per cent

between the first and third periods of bromide administration. The increase in the spinal fluid bromide ranged from 0 to 13.4 mg. per cent between the first and second periods of bromide administration and from 14 to 76.2 mg. per cent between the first and third periods of bromide administration.

The CSF protein levels showed decreases at the end of ACTH administration in 8 of the 9 cases ranging from 3 to 18 mg. per cent in 7 cases, and dropping 97 mg. per cent in Case 7. The CSF protein levels a week after stopping ACTH went up between 6 and 11 mg. per cent in 4 cases and dropped further between 6 and 12 mg. per cent in five cases. In all cases except the eighth there was some reduction of CSF protein level concomitant with ACTH administration.

Circulating eosinophils disappeared or were markedly reduced in all cases with the administration of ACTH. Sodium and chloride levels were not altered but potassium levels dropped in the seven cases where it was determined from 0.1 to 1.15 meq. per liter. There were no significant alterations in sedimentation rates.

DISCUSSION OF RESULTS

Clinically, no striking objective improvement was found in any case. In two cases where anxiety was quite evident a pseudo-improvement manifested by exaggerated cooperativeness was observed and seemed part of an attempt to deny the reality of a loss of function. No significant objective changes in improved strength, coordination, bladder function, or visual acuity that correlated with subjective claims were noted. One patient (Case 9) was profoundly depressed and had experienced a "remission" which he attributed to an earlier course of ACTH. He developed, with his second course, euphoria similar to that experienced before, and began making efforts that were previously not made. There was a slight apparent reduction in spasticity.

The series is not large enough to substantiate the ratio of CSF to serum bromide being elevated in multiple sclerosis. There is a suggestion in this direction, however. The ratios in the patients to whom ACTH was administered did not undergo significant or consistent alteration. This study did not include observations on the rate of penetration of bromide ions through the blood-CSF barrier.

SUMMARY

Nine male veterans suffering from multiple sclerosis were given ACTH by slow intravenous drip during a ten-day period. The permeability of the "blood-CSF barrier" before, during, and after the ACTH administration was studied by means of bromide determinations in serum and spinal fluid. There were no significant objective clinical changes noted during or after ACTH administration, although the drop in circulating eosinophils showed that the patients were reacting to the hormone. The changes in serum-CSF bromide ratios were variable and not marked during or after ACTH administration. There was some decrease in the amount of spinal fluid protein during ACTH administration in eight of the nine cases studied.

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CELLULAR CHANGES IN EFFUSIONS FOLLOWING INTRACAVITARY ADMINISTRATION OF COLLOIDAL Au^{198} IN HUMAN BEINGS

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THE effusions of serous cavities accompanying carcinomatosis have been treated recently with instillations of radioactive colloidal gold (Au^{198}). There is interest in the mechanism of action of this radioactive colloid, and in the fate of the colloid following its injection.

At the time of injection, the Au^{198} is a colloid having a submicroscopic particle size, estimated by different workers in ranges from 0.002 to 0.003 micron to 0.1 to 0.2 micron.[1] However, following its administration, it has been detected in particles and aggregates in the range of 1 to 5 microns in size. These are easily observed under a microscope, and have been seen by various workers. Sherman and associates[2] described gold particles which were phagocytosed by large mononuclear leukocytes, segmented neutrophils, and giant cells in patients who had been given parametrial injection of Au^{198}. Similarly, patients given intracavitary injections of Au^{198} and subsequently studied at autopsy were found to have aggregates of black pigment on or near the surfaces of treated cavities.[3] Particles were present both free and within phagocytes. Goldie and Hahn[4] have also observed phagocytosis of gold particles after intraperitoneal injection of colloidal radiogold into mice.

Factors causing the fluid accumulation in carcinomatosis are understood poorly and therapeutic action of gold^{198} in serous cavities is not well explained. Experimental work has demonstrated the effective treatment of free S-37 sarcomas in mice, but in treatment of more solid masses of tumor in human beings, alteration of tumor by colloidal Au^{198} is not expected, nor has it been demonstrated except on superficial portions of the treated surfaces.[5]

MATERIALS AND METHODS

This study is based on thirty patients who were treated with single or multiple instillations of radioactive colloidal Au^{198}. There were twenty-three females and seven males, and all had malignant tumors which were associated with persistent accumulations of ascitic or pleural fluid. The doses varied widely, since at the outset of the series multiple small doses of Au^{198} were given, while later the general plan was to give single large doses. Some of the patients received a series of rather large doses, and several were treated both in the pleural and peritoneal cavities. The largest single dose was 232 millicuries.

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The $gold^{198}$ used was a sterile colloidal solution* containing at the time of injection usually 10 to 25 millicuries per milliliter of radioactivity. The amount of stable gold present was generally in the range of 3 to 9 milligrams per milliliter.

Paracenteses for relief of symptoms were performed before and sometimes after instillation of the Au^{198}. In addition, in certain instances, samples of fluids were taken following the instillation of the colloidal gold, principally to determine the radioactivity. A total of 207 fluids were examined, the number of samples from a single patient varying from one to twenty-three, with intervals after treatment ranging from one day to six months. Cell counts, protein content, specific gravity, and volume were recorded in most cases. Aliquots of fluids were fixed in formalin, and cell blocks were prepared from centrifuged sediments. These were stained with hematoxylin and eosin, and were examined for total cellularity, malignant cells, and pigmented macrophages.

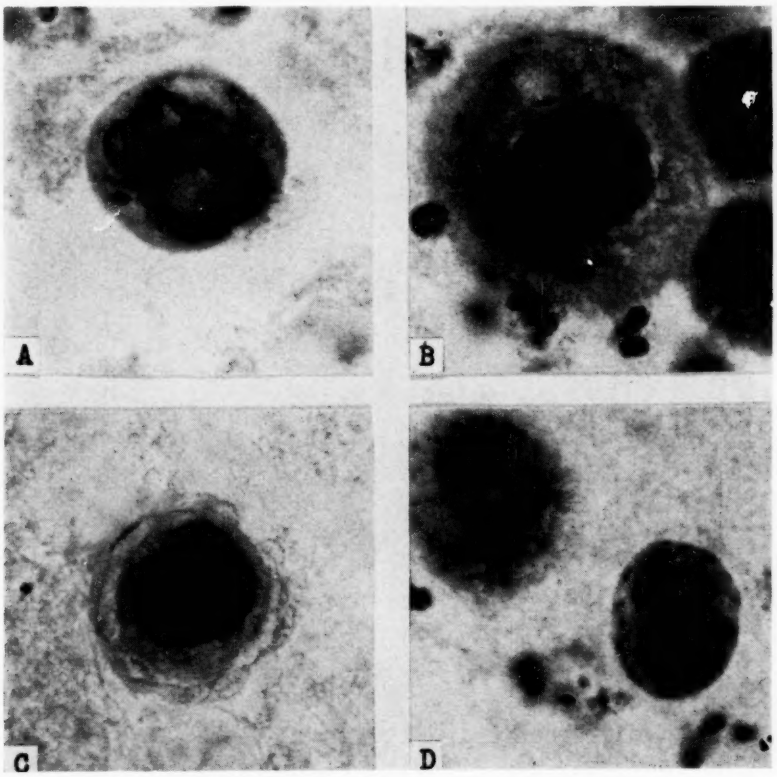

Fig. 1.—Malignant cells persisting in pleural fluid after injection of 155 mc. of colloidal Au^{198}. (×1164.) *A*, Six-day interval; *B*, twelve-day interval; *C*, eighteen-day interval; *D*, twenty-one-day interval.

This technique was adopted as a simple and practical method of obtaining information on the cell content of the fluids. More complete information, particularly on the presence of malignant cells, would undoubtedly have been obtained if more elaborate methods of cytologic diagnosis had been used.

RESULTS

Tables I and II indicate the presence or absence of malignant cells and gold-containing phagocytes, respectively, following therapy. In each case, the findings in subsequent paracenteses are tabulated according to the time interval

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TABLE I. MALIGNANT CELLS FOUND IN EFFUSIONS FOLLOWING THERAPY WITH Au^{198}

NO.	PATIENT	PRIMARY SITE OF NEOPLASM	DOSE AND SITE	MALIGNANT CELLS BEFORE RX	INTERVAL IN DAYS*									
					1-5	6-10	11-15	16-20	21-25	26-30	31-40	41-50	51-60	61-100	101-200
1	L. C.	Breast	55 mc. in 2 doses rt. pl.	++		+									
			40 mc. rt. pl.	+	0	0									
2	J. McG.	Uterus	232 mc. pe.	+0		+			?		0	00			
3	B. B.	Ovary	9.5 mc. in 2 doses pe.							+					
4	S. W.	Lung	155 mc. lt. pl.	+		+	+	++		0				+0	0
			148 mc. lt. pl.	+				0			+0	00			
5	C. F.	Pancreas	162 mc. rt. pl.	0			0				+		00		
			122 mc. pe.										+	0	
6	G. H.	Breast	210 mc. lt. pl.	0											
7	I. W.	Ovary	205 mc. pe.	+		+									
8	E. K.	Ovary	200 mc. lt. pl.	++0++	+		++	0	000	+		+			
9	P. N.	Not known	185 mc. rt. pl.	?	0			?			+				
10	E. F.	Ovary	170 mc. pe.	+		0	+								
11	J. C.	Ovary	196 mc. pe.				+				0				
12	C. R.	Lung	166 mc. lt. pl.	00			+								
13	M. D.	Lung	78 mc. rt. pl.	+			+								
14	P. B.	Lung	74 mc. rt. pl.	+		0		0							
15	H. R.	Ovary	101 mc. pe.	+		+		0	0	0	0				
16	M. B.	Not known	144 mc. pe.	++0		0									
17	O. S.	Breast	80 mc. rt. pl.	+000++00++++0			0	0	0	0		0			
18	M. G.	Ovary	105 mc. pe.	+0		0									
19	I. B.	Breast	78 mc. lt. pl.	+			0								
20	E. K.	Ovary	100 mc. pe.	00+		0	0					0	0		
21	R. H.	Breast	107 mc. pe.	+											

pl. = Pleura.
pe. = Peritoneum.
\+ = Positive.
0 = Negative.
*Interval in days from dose or last dose in a series.

NO.	PATIENT	DOSE AND SITE	INTERVAL IN DAYS*									
			1-5	6-10	11-15	16-20	21-30	31-40	41-50	51-60	61-80	81-100	101-200
1	L. C.	30 mc. rt. pl.		+									
		25 mc., 39 mc. rt. pl.	+						0	0			

NO.	PATIENT	DOSE AND SITE	INTERVAL IN DAYS* 1-5	6-10	11-15	16-20	21-30	31-40	41-50	51-60	61-80	81-100	101-200
1	L. C.	30 mc. rt. pl.		+									
		25 mc., 39 mc. rt. pl.	+										
2	J. McG.	232 mc. pe.			+	+	+	+	0	0			
3	B. B.	46 mc. in 7 doses pe. over 2 month period†	0										
4	S. W.	155 mc. lt. pl.		+	+	++	0						
		148 mc. lt. pl.										+	00
		22 mc., 17 mc. lt. pl.	+	+	+								
5	C. F.	162 mc. rt. pl.				+			0		00	0	
		122 mc. pe.											
		14 mc. rt. pl.		0									
		95 mc. rt. pl.		0									
		16 mc. rt. pl.	+		+		0						
		23 mc. rt. pl.		0									
6	G. H.	211 mc. lt. pl.			+	+	+	+					
7	I. W.	205 mc. pe.		+									
8	E. K.	200 mc. lt. pl.	0		00	0	+0+0+	+++0	00				
9	P. N.	185 mc. rt. pl.	+			+		+					
10	E. F.	169 mc. pe.		+	+								
11	J. C.	196 mc. pe.			+				0				
12	C. R.	166 mc. lt. pl.											0
13	M. D.	78 mc. rt. pl.			+								
14	P. B.	74 mc. rt. pl.		+	+								
15	H. R.	101 mc. pe.		+		+	00						
		49 mc. rt. pl.					+0						
16	M. B.	144 mc. pe.		+									
17	O. S.	80 mc. rt. pl.			+	+	++						
		3 mc. rt. pl.	+										
18	M. G.	105 mc. pe.			00								
		54 mc. lt. pl.		+									
19	I. B.	78 mc. lt. pl.			+								
20	E. K.	100 mc. pe.		+	+								
21	R. H.	107 mc. pe.							+				0
22	B. F.	6 mc. rt. pl.	+										
23	E. R.	60 mc., 100 mc., 100 mc. pe.				+		+					
24	G. C.	44 mc., 49 mc. lt. pl.		+									
		48 mc. lt. pl.		+									
		40 mc. lt. pl.							+		000		
25	A. M.	342 mc. in 6 doses pe.				0							
		52 mc. pe.	0			+							
		50 mc. pe.											000
26	C. C.	189 mc. pe.				+		0	0	00			
27	J. H.	185 mc. rt. pl.			+								
28	M. Z.	70 mc. lt. pl.		+									
29	L. R.	200 mc. lt. pl.			0	0	00	0+0			000		
30	D. O.	73 mc. rt. pl.					+						

pl. = Pleura.
pe. = Peritoneum.
0 = Negative for phagocytes containing Au pigment.
+ = Positive for phagocytes containing Au pigment.
*Interval in days from dose or last dose in a series.
† 12 specimens during period of therapy were negative.

after a single dose or the last dose of a series. A series consisted of two or more doses given in a relatively short period of time. In cases where two or more doses were given with long intervals between, each was considered separately.

Malignant Cells.—In fourteen of the thirty cases, malignant cells were found in the fluids following injection of the colloidal radiogold. Table I shows the dose and site of the injected Au^{198}, and the intervals in days between the injection and the aspiration of fluids. It is noted that tumor cells were seen at a rather wide range of intervals (see Fig. 1). This suggests that the radioactive gold does not rid the serosal surfaces of tumor even though clinical relief of fluid formation may be obtained.

Inflammatory Cells.—In evaluating the cellular exudation following administration of colloidal radiogold, we have studied the presence of phagocytes containing black pigment granules and the presence also of a variety of other inflammatory cells. It might be expected that the traumatic effect of the beta radiation on the peritoneal or pleural surface would excite an inflammatory reaction. This has been seen in a number of the fluids. Thirteen patients showed the presence of numerous polymorphonuclear leukocytes following the injection. However, five of these patients had similar findings in the fluid before therapy. When leukocyte counts were available they were over 2,000 per cubic centimeter, and the blocks were obviously cellular. In only one case was the inflammatory response after treatment believed due to pyogenic infection of the cavity. No clear pattern of reaction existed. The cellular response varied widely from patient to patient, and some individuals had remarkably few cells in the effusion after large doses. This is well demonstrated in Case No. 29, L. R., where 220 mc. were given into the left pleura. Following treatment, the histologic studies of the fluid showed few cells, and leukocyte counts ranged from 15 to 1,750 per cubic centimeter.

The general pattern of phagocytic activity shows an early appearance of gold-bearing macrophages in the fluid, the earliest positive sample having been obtained one day following the injection of the Au^{198}. These pigment-bearing phagocytes are seen rather consistently in the fluids but gradually decrease in number with the passage of time. Table II shows the time intervals following treatment when pigmented phagocytes were encountered in the effusion. In general, the fluids removed up to four or five weeks following therapy contained these cells, while most fluids after five weeks did not contain them. In cases where a series of fluids were available for study the number of phagocytes gradually decreased during the month following injection of the Au^{198}.

DISCUSSION

There are several possible explanations for the fact that the radioactive gold therapy does not consistently rid the serosal surfaces of tumor, even though retardation of fluid formation may be obtained. First, even though surfaces of pre-existing neoplasm may have been effectively irradiated, continued growth of implants beneath the surface will result in an invasion of the surface and will add a fresh source of exfoliating cells. The effective depth of radiation of

the Au^{198} is only a millimeter or so, and it has been demonstrated that the colloidal Au^{198} does not penetrate solid areas of neoplasm sufficiently to produce effective radiation of deeper tissues following the intracavitary injection.[5] A second possible explanation for the persistence of malignant cells is the fact that serosal surface distribution of the colloidal gold is uneven. An effort is made after injection of the gold to insure adequate mixing by shifting the patient about in various positions; but tissue radioassays at autopsy and surgical explorations show rather wide variations in concentration of radioactivity.[5] Another factor of importance in some cases is the presence of pockets of fluid which are partially walled off by fibrous or neoplastic adhesions. These pockets may not be reached by the gold instilled into the larger serosal cavity.

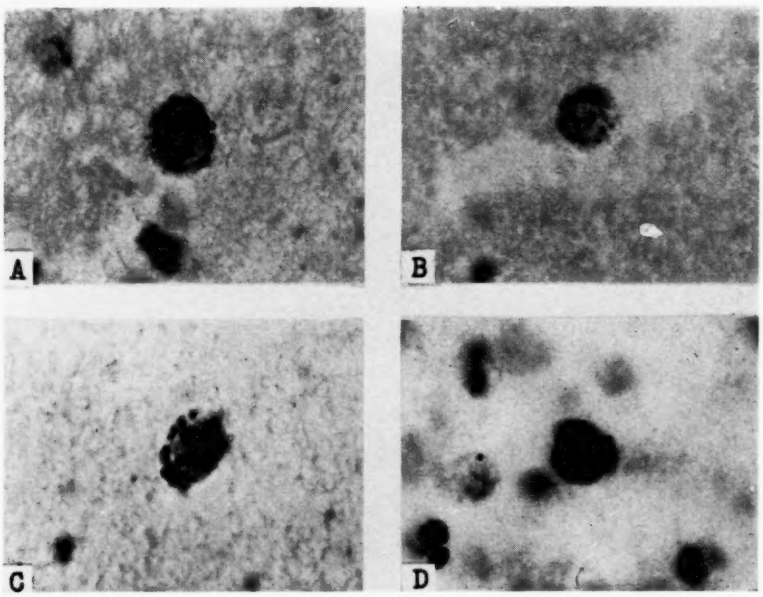

Fig. 2.—Phagocytes containing black aggregates of gold at varying intervals following injection into pleural space. (×1164.) *A*, Six days; *B*, twelve days; *C*, eighteen days; *D*, twenty-one days.

In untreated patients with effusions caused by neoplasms, the finding of malignant cells is rather unpredictable. Therefore, in cases where negative samples are obtained following treatment, we are hesitant to conclude that the therapy has caused the disappearance of malignant cells from the fluid. Some of these negative results appear to be simply chance variations in patients having fluids which were not consistently positive even before treatment. In addition, the samples studied following treatment were often smaller in volume. It is of interest that malignant cells persist in a significant number of the cases, and that clinical relief from rapid fluid accumulation was afforded some of these patients.

The small particles of gold which are found in aggregates within phagocytes are rather variable in size, but are estimated in the range from 1 to 2 microns.

Polymorphonuclear leukocytes apparently fail to phagocytose the Au^{198}, or disintegrate rapidly after the injection of the material, since only in rare instances are aggregates of pigment found in neutrophilic leukocytes. Goldie and Hahn[4] found in mice that macrophages were quite resistant to destruction by Au^{198} and, in contrast, polymorphonuclear leukocytes were highly sensitive. In twenty-four to forty-eight hours after injection, dotlike gold particles were found in phagocytes. They also noted that radioactive gold tended to aggregate in one or two large particles of irregular outline in the phagocytes, while in control animals given stable gold colloid, particles tended to be smaller, more numerous and dispersed. Our data from human cases of effusions do not show this tendency of the aggregates of Au^{198} to coalesce to the extent of forming only one or two large particles. Fig. 2 shows the appearance of these phagocytes in one case at varying intervals following the injection.

One of the chief problems still unsolved is the reason for the aggregation of the colloidal material into particles so large that they are easily visible under the microscope. Perhaps this is accomplished by the fusing of multiple smaller particles within the cytoplasm of phagocytic cells. Even though the large particles are extracellular in the walls of the treated cavities, it is possible that they previously originated within phagocytic cells which have subsequently disintegrated. On the other hand it seems to us more likely that these larger particles are formed as a result of the presence of protein or some other substance in the cavity.

SUMMARY

1. Pleural and ascitic fluids associated with malignant neoplasms have been studied in an effort to clarify the mechanisms involved in intracavitary radiogold therapy. Following treatment, malignant cells persist in a significant proportion of cases.

2. The inflammatory cellular response to the injection of the radioactive gold is quite variable.

3. Phagocytes containing gold pigment are found with regularity at various intervals following therapy, being seen as early as one day and usually present during the first four to five weeks after treatment. Subsequently, they are infrequently found.

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A RATIONAL APPROACH TO THE PROBLEMS OF THE COAGULATION TIME

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AS LONG ago as 1911, Cohen[1] collected thirty-one different means of estimating the clotting power or the coagulation time of blood. All of these were dependent upon drops of blood from skin punctures and the use of capillary tubes. Lee and White,[2] in 1913, in utilizing the venipuncture to avoid the rapid acceleration of the clotting process by the indiscriminate mixing of blood and tissue juice, were the first to make practical application of what physiologists had realized long before. Since that time, and particularly with the advent of the anticoagulants, much emphasis has been placed upon the detection of changes in the coagulation system. The large number of techniques and types of apparatus which continue to appear indicate the continuing dissatisfaction with available methods.

The estimation of coagulation time as ordinarily done is relatively insensitive and inaccurate. This has led some investigators to condemn the procedure as worthless.[3, 4] Poor results are in part due to failure to consider and control factors which influence the determination. The purpose of this study has been to consider and observe the influence of these factors and to attempt to control them in order to devise, if possible, a practical method of determining the clotting time which measures sensitively and accurately those degrees of hyper- and hypocoagulability which are of clinical significance.

FACTORS AFFECTING THE COAGULATION TIME AND MEASURES FOR CONTROLLING THEM

Ferguson,[5] in a recent review, has summarized the factors affecting coagulation. These are: the pH, the concentration or dilution of specific factors, the temperature, and the so-called "wettability effects" or physical changes produced by trauma and containers.

Hydrogen Ion Concentration.—The changes in pH during coagulation are minimal,[6] and do not deviate from a wide optimum. In the ideal test, however, no substances should be added which might influence the ionic balance or the pH.

Specific Factors.—The only addition of substance, and one of the chief objections in the direct method of determining coagulation time, is the unknown amount of tissue juice which is added to the sample during its extraction. With coagulation times of the Lee and White type, a venipuncture is used to decrease the effect of tissue juice. If one makes a venipuncture, aspirates a specimen, and then, without altering the position of the needle, changes syringes and withdraws a second specimen, an idea is obtained of the actual effect of the tissue juice. With skillful venipuncture there is little change unless the coagulation time is prolonged. With a

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traumatic puncture there is often a marked variation. In order to minimize the effect of tissue juice liberated at the time of venipuncture, we have adopted a two-syringe technique; in this method one first withdraws a small amount of blood to "clear" the needle of tissue juice, and then changes to a fresh syringe which is used for obtaining the specimen actually to be tested.

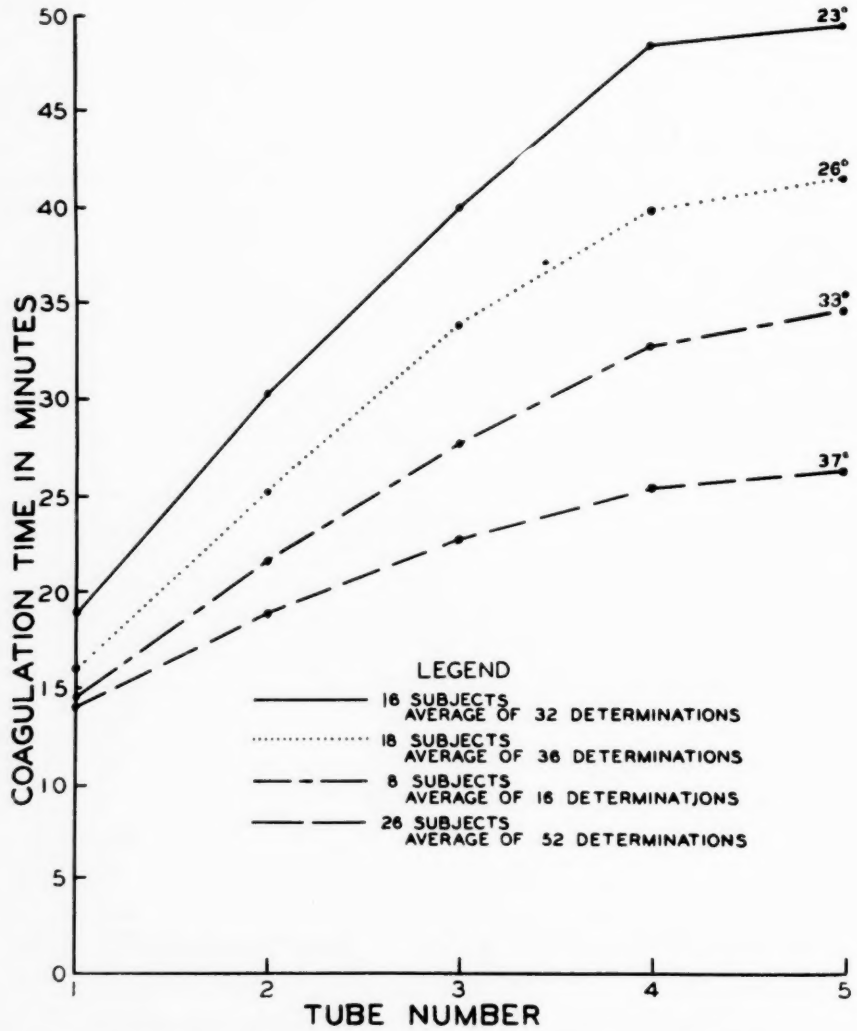

Fig. 1.—The effect of temperature upon the five-tube coagulation time.

Temperature.—Whittaker[7] has shown with the Waugh and Ruddick[8] test that variations in temperature can produce changes in clotting rates; our own studies, employing a multiple tube procedure with unheparinized blood, have demonstrated changes of similar order and magnitude. Fig. 1, derived from the results obtained in 68 healthy medical students, demonstrates the necessity of controlling temperature or correcting for it, if determinations of coagulation time are to be amenable to accurate interpretation. But the spread of the curves in Fig. 1 shows that the effect of temperature varies with the clotting rate, and suggests that it is more

feasible to perform the test at a constant temperature than to try to correct for this factor. We have therefore elected, not quite arbitrarily, to maintain the temperature of the blood near 37° C. during the period of the test.

Contact Factors.—Physical alterations produced in blood by contact with a foreign surface definitely influence the coagulation rate. This effect is illustrated by the ability of

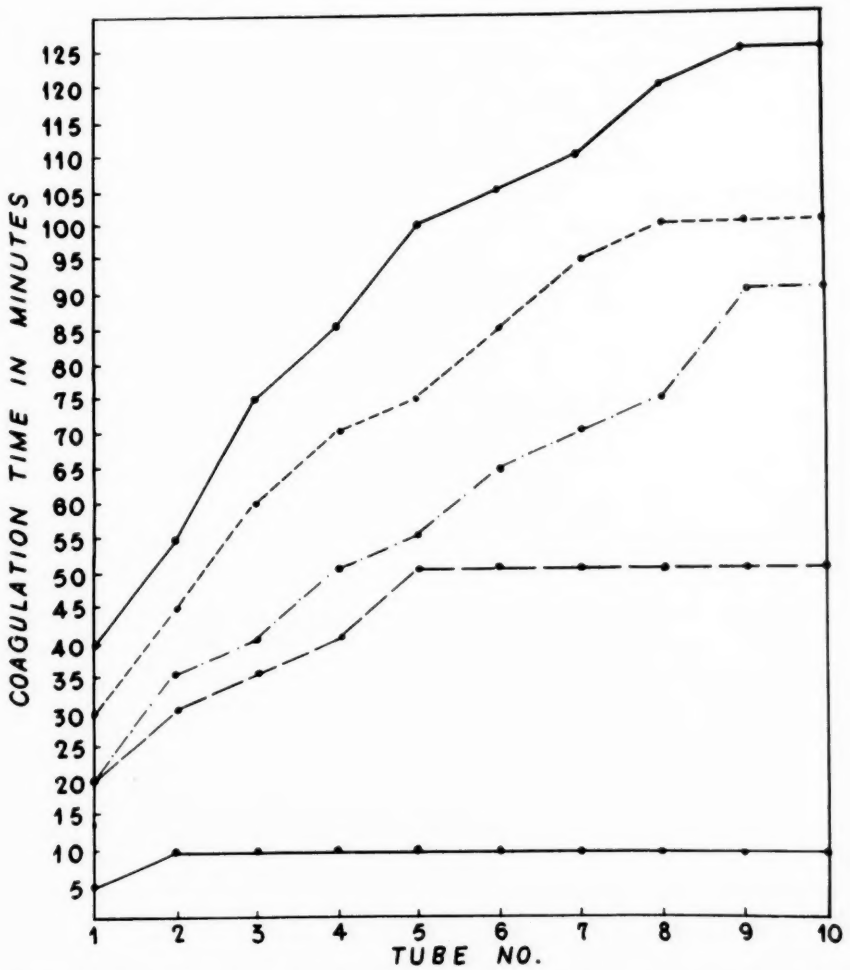

Fig. 2.—Examples of greater sensitivity gained by increasing the number of tubes in sequence to ten.

silicone,[9] collodion,[10] and other materials[11] to prolong the coagulation time. The mechanism of action is unknown, though it is said that the presence of foreign substances alters the first phase of coagulation.[12] Contact with glass affects clotting and is a source of variation which must be controlled.

Factors which determine the influence of glass are the size of the syringe; the manner in which the test tube is filled; the diameter and length of the tube; the frequency of tilting; and the manner of tilting. Individually these variables are of only small significance, but collectively they are a source of considerable error. It is obvious, therefore, that measures must be employed to "standardize" the effect of these physical factors, and to

reduce contact to a degree compatible with sufficient sensitivity. After considering the experiences of others and employing numerous modifications ourselves, we have adopted the following measures for the attainment of these ends:

(a) Glassware and needles are to be clean and dry. We do not use silicone coated materials as proposed by Mangulies and Barker,[9] for reasons to be given later. Nor do we advocate rinsing syringes and tubes with saline as in the original Lee and White method; moistening the apparatus does not appreciably diminish the "wettability" effect of glass, and merely introduces another indeterminate variable into the test.

(b) A 10 ml. syringe and a 20-gauge needle are used for withdrawing the sample; 10 ml. of blood are needed for the test, and a 20-gauge needle is the largest which is feasible for routine venipunctures. A larger, 18-gauge needle is used in transferring the blood to the test tubes, so as to minimize contact with the interior surface of the needle.

(c) The test tubes are filled from the bottom, so as to prevent violent contact with their surfaces.

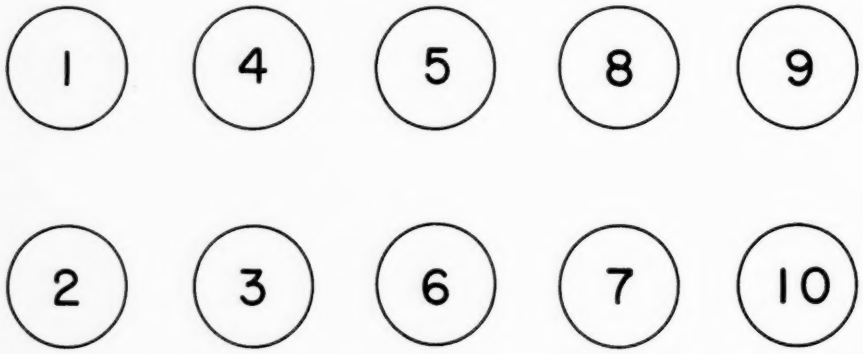

Fig. 3.—The order of filling tubes so as to obtain equality in time of glass exposure between the duplicate tests.

(d) Relatively large caliber, short tubes are used (internal diameter 9.5 mm., height 40 mm.). In smaller tubes there is a relatively greater blood-glass interface and resultant reduced sensitivity; longer tubes are more difficult to fill, and present a greater surface for contact as the tube is tilted.

(e) In examining for the end point, tubes are completely inverted every five minutes. More frequent inversion, by allowing additional exposure to glass, decreases sensitivity and may, if coagulation is delayed, cause partial defibrination with resultant incomplete clotting. Complete inversion rather than mere tilting insures uniform contact with glass each time the unclotted blood is disturbed.

(f) A five-tube technique with duplicate determinations is recommended. The value of multiple tube techniques in reducing glass effect and increasing sensitivity is well known, and clearly illustrated in Fig. 2, derived from a study of five selected cases, in one of which the blood was hypercoagulable, the others showing varying degrees of hypocoagulability. At tube 1 the spread between the shortest and the longest clotting time is thirty-five minutes; at tube 5 it is ninety minutes; at the tenth tube, one hundred fifteen minutes. It is our present opinion, reached after experience with ten-tube tests in many cases, that variations beyond the fifth tube are of little or no clinical significance.

THE METHOD

The equipment must be clean and dry. A tourniquet is applied above the elbow and left in place. The site of puncture, preferably in the antecubital area, is cleansed with an antiseptic, and the venipuncture is made as atraumatically as possible with a 20-gauge needle attached to the preliminary syringe. At least 2 ml. of blood are withdrawn to clear

the needle of tissue juice released during the puncture. With the needle left in place, a second clean syringe is attached, and slightly more than 10 ml. of blood is aspirated. Foam and change of needle position are avoided. The tourniquet is released and the needle withdrawn. An 18-gauge needle is then attached to the syringe containing the testing specimen. Agitation of the blood is avoided. One milliliter of blood is gently and evenly ejected without bubbling into each of ten soft glass test tubes (internal diameter 9.5 mm., height 40 mm.) by placing the needle near the bottom and filling the tube from below upward. The tubes are stoppered with number 00 rubber corks and are racked as shown in Fig. 3. The rack and tubes are placed in a portable water bath at 37° C.

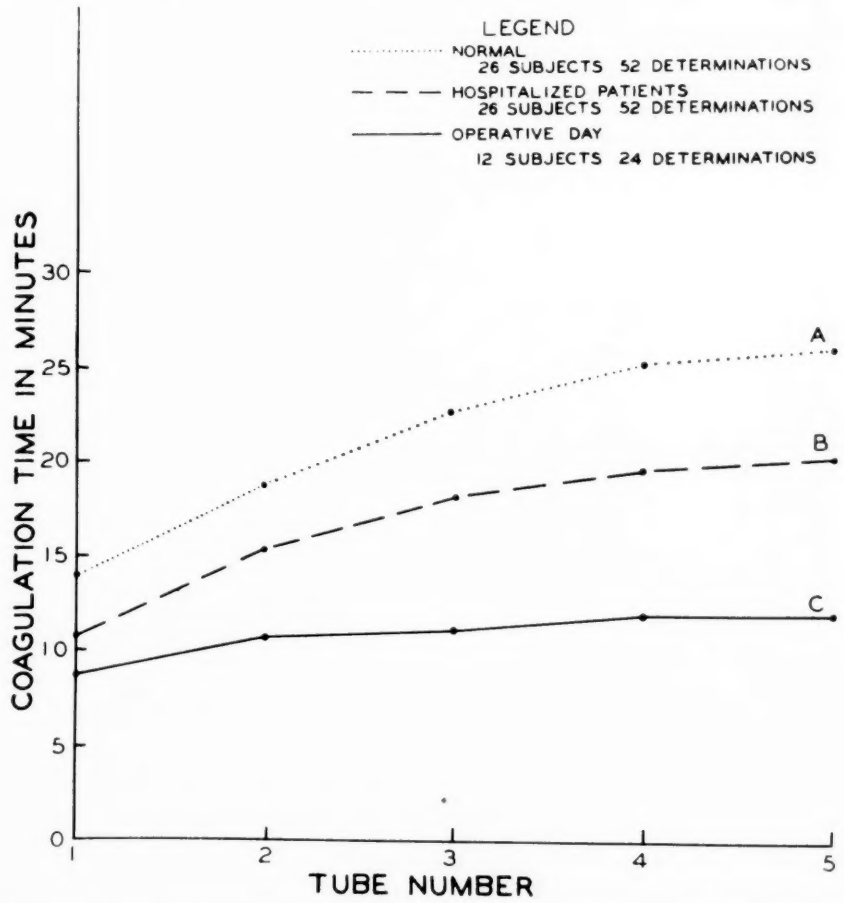

Fig. 4.—The coagulation times of normal, hospitalized, and postoperative subjects.

Timing is begun at the appearance of blood in the second syringe. Tubes 1 and 2 are examined after five minutes and at five-minute intervals thereafter until the end point is reached. To determine the end point the tube is completely inverted, and, if there is no flow, is gently tapped two or three times. If the blood does flow, it should be allowed to do so completely so as to obtain equal glass exposure at each examination. If there is no flow after tapping the inverted tube, then the end point has been reached. The only factor of uncertainty in determining this end point is the occasional appearance of small amounts of serum. If clotting is incomplete, the tube is returned to the water bath and re-examined in five minutes. When the end point has been reached the tube is discarded and the next

in sequence is examined immediately, as for instance 1 to 4, then 4 to 5, and so on. No tube is discarded until clotting is complete. The final result is the average of the duplicate determinations, that is, the average of tubes 9 and 10.

RESULTS

The Coagulation Time of Normal Subjects.—Curve *A* of Fig. 4 demonstrates the average coagulation time in 52 determinations on 26 normal medical students. The points on the curve represent the average value of the coagulation time in each of the five tubes, the fifth point being the final value, twenty-six minutes. Bar graph *A*, Fig. 5, is the frequency distribution of these same determinations as it occurs in the fifth tube. It is to be noted that 96 per cent of

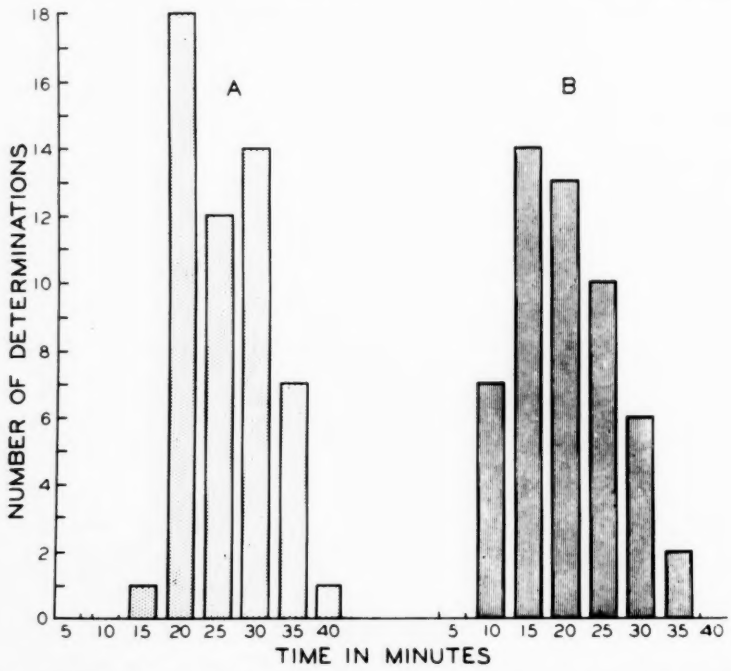

Fig. 5.—Frequency distribution of coagulation times of normal and hospitalized subjects.

these fall within the range of twenty to thirty-five minutes. In only one subject, an incidence of 2 per cent, was the coagulation time below twenty minutes, and in no subject was it below fifteen minutes. Similarly, in only one subject (2 per cent) was the determination above thirty-five minutes, and in none above forty minutes. We have therefore accepted as normal a range of twenty to thirty-five minutes.

The Coagulation Time of Hospitalized Patients.—Curve *B*, Fig. 4 represents 52 determinations on 26 hospitalized patients. These patients, for the most part ambulatory, had no acute illnesses, but were awaiting interval surgery. The average coagulation time of 20.3 minutes, 5.7 less than the normal average, is statistically significant. Bar graph *B*, Fig. 5, is the frequency distribution of the determinations in these patients. We do not know at this time what part

age and sex differences might have made in this apparent shift toward hypercoagulability. We know only that incident to hospitalization the physical activity of these subjects was considerably restricted.

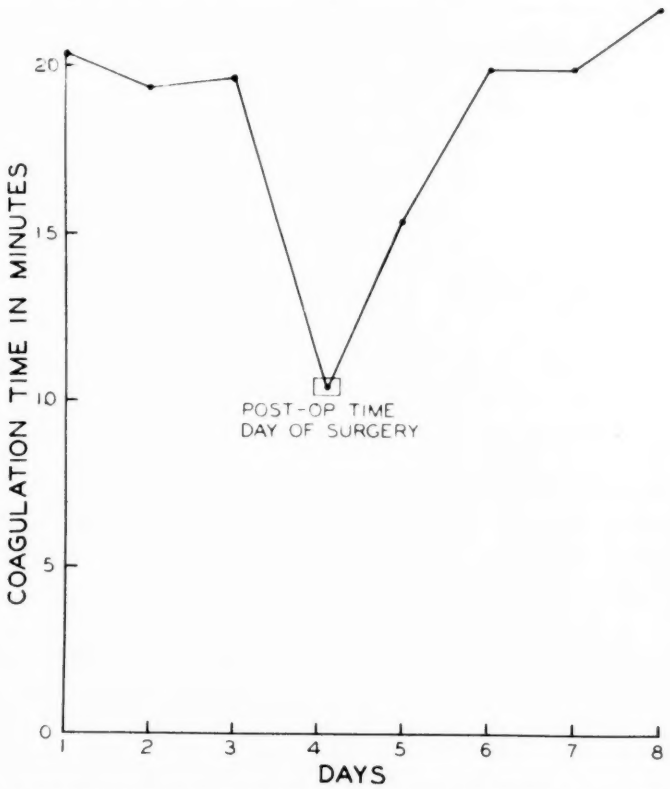

Fig. 6.—The average coagulation times of ten consecutive patients before and after major surgery.

The Effect of Surgery on Coagulability.—Curve *C*, Fig. 4 represents the average of the coagulation times obtained in 12 consecutive patients within twenty-four hours after major surgery. Fig. 6 demonstrates further the hypercoagulability occurring after major surgery. The significant average fall in coagulation time in the 10 consecutive subjects whose determinations form this graph becomes even more striking when one considers the fact that two patients with relatively atraumatic surgery showed no drop at all.

DISCUSSION

The modifications which have been proposed show promise of measuring levels of coagulability with accuracy and sensitivity heretofore unobtainable with the Lee-White techniques. Others have demonstrated increased sensitivity in detecting clotting changes by the use of the heparin tolerance principle or coated materials.[7, 9] The use of heparin to detect changes has several inherent difficulties. First, there is considerable variation in heparin potency which requires

some form of biological standardization.[13, 15] Second, misleading changes may be produced by the addition of such a powerful anticoagulant. Third, there is a wide range of individual response to heparin. And fourth, the described sources of error in doing the clotting time are still present.

There is no question of the ability of coating material such as silicone to add sensitivity to a clotting determination, but the relative error remains the same. This procedure increases the expense and the time required for performing the test.

The occurrence of thrombosis or hemorrhage depends upon much more than the immediate clotting power of blood.[16] The smoothness of the blood vessels, the intravascular pressure and flow rate, the reserves of the coagulation system, the degree of stasis, the changes in hemoconcentration or dilution which may take place, and the amount of injury or release of tissue juice and other substances at a time of trauma, are all factors which can determine hemorrhage or thrombosis. We are definitely limited, therefore, in the information which we can obtain regarding prediction, prevention, and control of such instances by a coagulation test. Fortunately there is a wide range throughout which the factors which influence coagulation may operate with a minimum of incidents; however, beyond this favorable range at both ends of the spectrum, these incidents become increasingly common. It is to increase further the clinical feasibility of measuring these outer ranges that the modifications described are proposed.

SUMMARY

A modified Lee and White method for determination of coagulation time is described and proposed, which is believed to be more accurate and more sensitive than the methods currently in use, and which is not overly complicated or time-consuming. The method is designed to standardize and, insofar as is feasible, to minimize extraneous factors which influence the coagulation of venous blood.

Application of the method in the study of normal subjects, hospitalized patients awaiting elective surgery, and postoperative surgical patients is reported.

It was found in normal subjects that the average coagulation time by the proposed method was twenty-six minutes, with a range of twenty to thirty-five minutes. A statistically significant decrease in the average coagulation time in hospitalized patients awaiting surgery was observed, and a further drop was demonstrated on the first postoperative day.

It is emphasized that due to the multiplicity of factors which affect intravascular clotting, no coagulation test can invariably indicate predisposition to thrombosis or hemorrhage, but it is hoped that the proposed method will increase the probability of predicting such occurrences.

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A STUDY OF THE RAPID SLIDE AGGLUTINATION TEST FOR BRUCELLOSIS AND A COMPARISON WITH THE TUBE AGGLUTINATION TEST

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THE rapid macroscopic slide agglutination test for the serum diagnosis of Bang's abortion disease of cattle, developed and used first by Gwatkin,[1] has been studied and employed extensively by workers in the field of veterinary medicine. The macroscopic slide or plate agglutination test has been applied also to the laboratory diagnosis of human brucellosis, using either antigens prepared in the laboratory or those obtained from commercial sources. Many public health laboratories, whose daily volumes of brucella agglutination tests are considerable, have used the rapid slide technique either as a screening device to select specimens which should be confirmed by the slower tube agglutination test or as a substitute for the tube test. The rapid slide agglutination test developed by Castaneda[2] to be made directly on the patients' whole blood was not considered in this study.

A comparison of the Huddleson rapid slide test[3] and the Bureau of Animal Industry slide test[4] indicates that the principal differences are concerned with (a) the strains of *Brucella* employed, (b) the antigen densities, and (c) the concentrations of saline. For example, Huddleson and Abel,[5] in discussing the preparations of antigen for the rapid macroscopic slide agglutination test, state that "the more concentrated the antigen, the more satisfactory it will be," whereas Donham and Fitch[6] conclude that "the less the bacterial density of the antigen, the higher the agglutination titer." The latter authors stated also that no significant differences in sensitivity could be detected in sodium chloride concentrations ranging from 0.85 to 15 per cent. A study of the brucella agglutination titers obtained by various laboratories on a series of test sera was made by Eisele, McCullough, and Beal[7] who postulated that the differences in titer may have resulted from a lack of standardization of the antigens employed.

The present study was undertaken to evaluate the slide test and to compare the results obtained with the slide procedure and the tube agglutination procedure using sera from cases of human brucellosis.

EXPERIMENTAL

Human sera were obtained through the State departments of health of several states in which brucellosis occurs with some frequency. The sera were from patients either with culturally proved brucellosis or from clinically suspicious cases with brucella tube agglutination titers reported as 1 to 40 or higher. In view of the incomplete histories relating to the specimens, all sera will be referred to as brucella-reacting sera.

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The tube tests, which employed antigen prepared from NIH Brucella strain 456 suspended in 0.5 per cent phenolized saline and adjusted to a McFarland density Standard No. 1, as described in a previous study,[8] were read after twenty-four hours' incubation in a water bath at 37° C. in 0.5 per cent phenolized saline. Concentrated suspensions for the slide test were harvested from Roux bottles containing tryptose agar[8] after seventy-two hours' incubation at 37° C. The growth was removed in 0.5 per cent phenolized saline, filtered through cotton, and heated in flowing steam for one-half hour. The organisms then were centrifuged and resuspended as a concentrated suspension in 0.5 per cent phenolized saline. The density for each antigen was adjusted separately, after which the volume of cells was determined with Hopkin's vaccine centrifuge tubes; 0.2 ml. of the concentrated antigen was diluted with saline to approximately 5.0 ml. and centrifuged for seventy-five minutes at 2,750 r.p.m. (standard 30 centimeter centrifuge head). The average reading of 4 vaccine centrifuge tubes was multiplied by five to obtain the equivalent reading for 1 ml. and then by 100 to express as per cent. Brilliant green and gentian violet were added to the antigen as bacteriostatic agents in accordance with the directions shown in Diagnostic Procedures and Reagents.[9] Glycerin (20 per cent by volume) was added to the antigens to prevent rapid drying of the mixture on the slide.

The tests were carried out on the Minnesota rapid slide testing box.* Quantities of 0.08, 0.04, 0.02, 0.01, and 0.005 ml. of each serum were placed on five separate squares of the glass plate and 0.03 ml. of antigen was added to each quantity of serum. Such serum dilutions or ratios were reported by Noble[10] and by Huddleson and Abel[5] to give results which compared empirically to tube agglutination titers of 1 to 20, 1 to 40, 1 to 80, 1 to 160, and 1 to 320. Higher dilutions were prepared by mixing one part of serum with 15 parts of 30 per cent bovine albumin diluent, again placing 0.08, 0.04, 0.02, 0.01, and 0.005 ml. of the diluted serum on the slides and mixing with 0.03 ml. of antigen. Higher dilutions were obtained by repeating the procedure.

Each serum-antigen mixture was stirred with a separate applicator and allowed to stand for five minutes, after which the glass plate was tilted back and forth with a rotary motion for approximately thirty seconds. The readings were made at the end of eight minutes. In both the tube and the slide test, the titer was recorded as the smallest quantity of serum which showed the agglutinated antigen in a clear supernatant fluid.

The brucella macroscopic agglutination tube tests were compared with those obtained with slide tests using antigen densities of 2.5, 5, and 10 per cent suspended in 0.85, and 12 per cent saline. The 10 per cent antigen in 0.85 per cent saline follows the recommendations of the Bureau of Animal Industry. An additional antigen was made with 13 per cent cells suspended in 12 per cent saline to simulate the procedure recommended by Huddleson.

RESULTS

For convenience, the data are presented in two parts as follows: (1) The results obtained with low-titered sera (to evaluate the slide test as a screening procedure) and (2) results obtained with all sera (to evaluate the slide test as a possible substitute for the tube test in determinating titer).

The data pertaining to the use of the slide test as a screening procedure are presented in Table I, which gives the results obtained with low-titered sera (1 to 80 or less in the tube test). From inspection of the table, it is obvious that lower titers are obtained with the higher concentrations of antigen. The differences in titer obtained with saline concentrations of 0.85 and 12 per cent are not so marked, but, in general, higher titers are obtained when the organisms are suspended in 0.85 per cent saline. The most important observation, from the viewpoint of the slide test as a screening device, is the number of

*Purchased from Jen-Sal Laboratory Supply House, Kansas City, Mo.

false negative reactions obtained with the more concentrated antigen. Of 13 sera giving tube titers of 1 to 40, 2 gave negative slide tests with the 10 per cent antigen and 3 gave negative results with the 13 per cent antigen. The discrepancy was more marked with low-titered sera (1 to 20 in the tube test). Here the data indicated that false negatives were not encountered when 5.0 per cent antigen was employed and that the concentration of saline made little difference.

TABLE I. COMPARISON OF VARIOUS ANTIGEN DENSITIES AND SALINE CONCENTRATION FOR THE SLIDE AGGLUTINATION TEST WITH LOW-TITERED SERA

PER CENT ANTIGEN DENSITY	NEG.	POSITIVE					NEG.	POSITIVE				
	0.08	0.08	0.04	0.02	0.01	0.005	0.08	0.08	0.04	0.02	0.01	0.005
	SLIDE TITERS											
	0.85% SALINE						12% SALINE					
	13 Sera Giving Tube Titers of 1:80											
2.5				9	3	1			1	10	2	
5.0			1	10	2				7	6		
10.0			9	4				2	7	4		
13.0								5	8			
	13 Sera Giving Tube Titers of 1:40											
2.5			2	10	1				7	5	1	
5.0			9	4				1	9	3		
10.0		6	6	1			2	7	4			
13.0							3	7	3			
	5 Sera Giving Tube Titers of 1:20											
2.5	1		3	1				3	1	1		
5.0		2	2	1				3	2			
10.0	3	2					3	2				
13.0							4	1				
	19 Sera Giving Negative Tube Agglutination in 1 to 20 Dilution of Serum											
2.5	4	1	4	6	4		2	2	5	9	1	
5.0	1	2	8	7	1		3	2	9	5		
10.0	3	4	8	4			3	5	10	1		
13.0							4	8	6	1		

On the other hand, the slide test yields too many positive agglutinations with sera which give negative reactions by the tube test. The percentages of false positive reactions (80 to 95 per cent) are not related to the concentrations of the antigen or the saline content.

The results of the tube test titers obtained over the whole range of serum dilutions and the titers obtained with 3 of the slide test antigens (BAI, Huddleson, and 5.0 per cent antigen in 12.0 per cent saline) are presented in Table II. Although there are no significant differences between the slide test titers obtained with these various antigen dilutions, the data show that the 5.0 per cent antigen is just as satisfactory as the more concentrated antigens.

DISCUSSION

The data show that higher titers are obtained with the slide test when low antigen densities rather than high antigen densities are employed. Similarly, low salt concentrations increase the sensitivity of the slide test, whereas high salt concentrations decrease it. However, it is not possible merely by picking the most sensitive combination of salt and antigen to select a slide test antigen

TABLE II. COMPARISON OF THE *Brucella* TUBE AGGLUTINATION TEST WITH THE SLIDE AGGLUTINATION TEST TUBE USING DIFFERENT ANTIGEN SUSPENSIONS
TUBE AGGLUTINATION TITERS

SLIDE AGG.	SERUM	LESS 1/20	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	SLIDE TITERS*
	Neg. .08 ml.											1/20 less
5.0%	.08 ml.	3										1/20
Antigen	.04 ml.	2	3	1								1/40
12.0%	.02 ml.	9	2	9	7							1/80
Saline	.01 ml.	5		3	5	10						1/160
	.005 ml.				1	5	1					1/320
	(1-15) .08 ml.					1	1					1/640
	(1-15) .04 ml.					2	3	7	4			1/1280
	(1-15) .02 ml.							4	3			1/2560
	(1-15) .01 ml.							1	1	4	2	1/5120

	SERUM	LESS 1/20	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	SLIDE TITERS
	Neg. .08 ml.											1/20 less
	.08 ml.	3	3									1/20
	.04 ml.	4	2	6								1/40
10.0%	.02 ml.	8		6	9	2	1					1/80
Antigen	.01 ml.	4		1	4	11						1/160
0.85%	.005 ml.					3	3	3	1			1/320
Saline	.08 ml.					2	1	7	4			1/640
(BAI	.04 ml.							2	3	2	1	1/1280
Test)	.02 ml.									2	1	1/2560
	.01 ml.											1/5120

	SERUM	LESS 1/20	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	SLIDE TITERS
	Neg. .08 ml.	4	4	3								1/20 less
	.08 ml.	8	1	7	5							1/20
13.0%	.04 ml.	6		3	8	7						1/40
Antigen	.02 ml.	1				8	1					1/80
12.0%	.01 ml.					2						1/160
Saline	.005 ml.					1	3	6	2			1/320
(Huddle-	.08 ml.						1	6	5			1/640
son Test	.04 ml.								1	1		1/1280
Antigen)	.02 ml.									3	2	1/2560
	.01 ml.											1/5120

*Equivalent Huddleson slide titer.

which compares best with the tube test titers. For example, the most sensitive combination in the series (2.5 per cent antigen in 0.85 per cent saline) showed the poorest agreement with the tube test. This probably may be explained by considering that the titers reported with the rapid slide test are not true dilutions of 1 in 20, 1 in 40, etc., but rather represent ratios obtained when serum quantities of 0.08, 0.04, etc., are mixed with 0.03 ml. of antigen whose reactions are designed to correspond with the respective tube dilutions.

Although the Castaneda[2] antigen as studied by Spink and Anderson[11] was so titrated that positive reactions were obtained only when the antibody was present in a tube titer of 1:100 or above, they obtained lower titers and even negative results in many instances when a significant titer of *Brucella* agglutinins were detected by the tube dilution method. It is felt that such results are inevitable when the sensitivity of the test is adjusted so that it will react only at an arbitrarily-selected diagnostic antibody titer.

The data in Table I show that, regardless of the antigen density, from 15 to 18 sera were positive with the slide test, and gave negative reactions in the

tube test. This may be explained by the fact that the slide test made with 0.08 ml. of serum and 0.03 ml. of antigen represents a serum dilution of only 1 in 1.37.

The *Brucella* strains for antigen preparation, as employed by Huddleson, and by the Bureau of Animal Industry Laboratories, were not used in the preparation of antigens for this study, because of the results of a previous investigation[5] in which it was shown that neither the Bureau of Animal Industry strain 1119-3 nor the Huddleson strain 1336 was superior to the NIH strain 456 for the preparation of brucella agglutination antigen.

The dilutions above 1 in 320 were made by diluting the specimen 1 to 15 according to the Bureau of Animal Industry method.[4] However, in this study the 30 per cent bovine albumin was used as the diluent instead of negative reacting bovine serum.

SUMMARY AND CONCLUSION

Sera from 99 patients with proved or presumptive brucellosis were studied in this laboratory for the purpose of evaluating the rapid slide test procedure as a possible substitute for the tube test method of detecting antibodies in hospital and public health laboratories.

Although all sera were reported to have had tube test titers when first examined, some loss of reactivity occurred by the time the present tests were done, 24 of them having titers of 1 to 20 or less including 19 sera which gave no reaction in the 1 to 20 dilution of serum.

Antigen concentrations of 2.5, 5.0, 10, and 13 per cent in 12 per cent saline, and 2.5, 5.0, and 10 per cent in 0.85 per cent saline were used in the slide test and the results compared with those obtained by the tube test using an antigen of McFarland No. 1 density, incubated for twenty-four hours at 37° C.

Over the whole range of serum dilutions, the antibody titers were depressed as the densities of the antigen were increased. The concentration of saline made little difference, but, in general, more consistent results were obtained with the higher saline concentration.

The 5.0 per cent antigen was definitely superior to the 10 and 13 per cent antigens when tested with low-titered sera in that no false negative reactions were encountered.

As a screening test in the routine laboratory, it is suggested that the slide test be performed with 5.0 per cent antigen suspended in 12 per cent saline to test 0.08, 0.04, 0.02, 0.01, and 0.005 ml. of serum. The full series is recommended in order to avoid reporting as negative a high-titered serum with a marked prozone. A serum giving negative reactions in all dilutions can be reported safely as negative, but a positive reaction in the slide test does not signify that the tube test will be positive.

The slide procedure using the 5.0 per cent antigen as tested in this study can be used as a satisfactory substitute for the tube test procedure to determine the titer of a serum.

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SOME OBSERVATIONS ON THE ASSAY OF STAPHYLOCOCCAL ENTEROTOXIN BY THE MONKEY-FEEDING TEST

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THERE is at present no in vitro test available for detection of staphylococcus enterotoxin. Of the animal test methods available (other than feeding of human volunteers), the two which seem to be most useful are (1) the intravenous injection of cats with culture filtrates in which nonenterotoxic components have been rendered innocuous, and (2) feeding of young monkeys with culture supernatant fluids.[1-4]

The intravenous cat method requires more negative control tests than the monkey feeding method. Toxic materials other than enterotoxin which may provoke emesis when administered by parenteral routes must first be rendered inactive by the use of heat, formalin, or antiserum.[2] Each of these procedures may have some disadvantages for experimental work. It is generally agreed that heating at 100° C. for twenty to thirty minutes probably inactivates part of the enterotoxin. It might be expected that the rate of inactivation of enterotoxin would vary at different stages of purification depending on the amounts and kinds of other materials (organic and inorganic) present in the sample at the time of heating. The use of formalin (which requires holding the sample in solution at 37° C. for several days) might result in similar sources of error. Serum neutralization is based on the assumption that antiserum prepared against toxic materials from nonenterotoxigenic strains will neutralize all such materials other than enterotoxin which may cause vomiting by this route. This apparently is true and subject to control (by titration of alpha and beta toxins) when working with crude toxins; however, trouble might be anticipated if some other toxic factor were concentrated along with the enterotoxin. Although some of these objections to the parenteral route may be theoretical, it would be extremely difficult to devise adequate controls to eliminate these possible sources of error. Therefore, when the present investigation[5-8] of the chemical nature of enterotoxin was undertaken, it was decided that the oral monkey test would be the most reliable assay method for use in purification studies.

During the course of this investigation the frog test,[9, 10] recently described for detection of enterotoxin, has been found to be unreliable in our hands. Nineteen intact and 85 decerebrated frogs (*Rana pipiens*) were fed enterotoxin or various nontoxic control materials. The reactions of frogs to enterotoxin were inconsistent and apparently unrelated to dosage. Positive

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reactions (reverse peristaltic waves) have been seen in frogs fed saline and other nonenterotoxic materials. These results are in agreement with those of Eddy[11] and Tekse.[12]

The purpose of this report is to present certain observations which have been made in connection with the use of monkeys for assay of staphylococcus enterotoxin by the oral route. These may have some bearing on the interpretation of monkey feeding tests made to determine the enterotoxigenicity of staphylococci isolated from foods involved in outbreaks of food poisoning.

MATERIALS AND METHODS

Most of the work has been done with the staphylococcus strain 196 and a variant 196E; some with strain S-6 and also a few other strains described in previous reports.[13, 14] Strain 196 was isolated in 1940 by Dr. G. G. Slocum from ham involved in a food-poisoning outbreak. Strain S-6 was isolated (1947) in small numbers from wholesome frozen shrimps by Dr. J. Evans.

The material assayed was crude or partially purified enterotoxin.[5, 6] The monkeys used were young *Macaca mulatta*.

Enterotoxin was assayed by the monkey feeding test. The toxin, usually in 50 ml. volume, was given by stomach tube. The animals were then observed continuously for five hours. Although ingestion of enterotoxin causes various symptoms,[1] only vomiting was accepted as a positive test for enterotoxin.

EXPERIMENTAL RESULTS

Susceptibility of Animals.—Most of our data on the assay of enterotoxin in monkeys has been obtained in connection with attempts to purify the toxin.[6, 8, 15] When toxic solutions were fractionated to give several samples, these were fed in equal doses on a volume basis (in terms of crude toxin) to determine which contained most of the enterotoxin. Therefore, there was considerable variation in dosage of toxin fed. No attempt has been made to determine 50 per cent emetic doses when comparing enterotoxic fractions. Whenever possible, concentrated toxins have been assayed by feeding each of two or three concentrations (of fivefold differences) to 6 monkeys. Since it is necessary to use animals several times, an attempt has been made to include in each group of six 3 "susceptible" and 3 "resistant" animals. Monkeys were classified as susceptible or resistant on the basis of past reactions to enterotoxin. Animals were considered resistant when they failed to vomit, while others fed the same dose of toxin gave positive reactions; new monkeys fed for the first time were considered susceptible. This classification involves some subjective interpretation of past feeding records due to the wide range of potency of samples fed. Representative results of 274 feeding tests made during the first four months of 1950 are presented in Table I (only data from toxic samples are included). The toxins were derived from several strains of staphylococci; however, most of the work was done with one strain, No. 196E. The incidence of vomiting reactions was 59 per cent in susceptible and 28 per cent in resistant animals. Animals which never vomited when fed enterotoxin have been excluded from the table; inclusion of these would lower the vomiting incidence in the resistant group. Although all the animals included in the tabulation gave at least one positive reaction at some time, it is never-

TABLE I. REPRESENTATIVE VOMITING REACTIONS OBTAINED IN "SUSCEPTIBLE" AND "RESISTANT" MONKEYS*

SAMPLE NO.	ML. FED†	NO. POS./NO. FED	
		SUSCEPTIBLE	RESISTANT
4	40	1/3	0/3
	200	3/3	1/3
	1000	2/3	2/2
7	40	1/3	0/2
	200	2/3	2/2
	1000	2/3	0/2
9	200	1/3	0/3
	1000	3/4	0/2
		15/25	5/19
		60%	26%

*Monkeys were classified as "resistant" on the basis of negative reactions to doses which caused other monkeys to vomit.

†Crude toxin equivalent (volume of crude toxin from which the toxin was obtained).

Totals from 1/11/50 to 5/19/50

Susceptible 92/155 = 59.4 per cent.

Resistant 33/119 = 27.7 per cent.

theless probable that differences in initial susceptibility may account for part of the difference in incidence of emesis, acquired resistance accounting for the remainder. It is emphasized that the assays yielding the above figures were not designed to obtain precise information on acquired resistance, but rather to evaluate the relative potency of enterotoxic fractions. These figures would vary considerably depending on the toxin dosage, rate of turnover of animals, and perhaps other unknown factors.

Studies on the stability of enterotoxin under different conditions of storage have been hampered greatly by the inability to reproduce assay results. Two examples of the wide variation in emetic response obtained at different times with the same samples are given in Table II. Lyophilized samples No. 91 and No. 92 which were produced and fed in February with negative results gave positive results when fed at the same dosage three or four months later. The quality of animals (with regard to past feeding records) was apparently similar at the various feeding times. Numerous such

TABLE II. VARIABILITY OF EMETIC RESPONSE TO ENTEROTOXIN. MONKEY FEEDING TESTS

SAMPLE NO.	DATE	DOSE		NO. POS./NO. FED
		MG.	ML.*	
91	2/14/51	1.44	39	0/6
	2/14/51	7.2	195	0/6
	5/25/51		195	4/6
	7/ 6/51		195	0/6
	7/10/51		195	1/6
	3/ 4/52	7.4	200	1/6
92	2/23/51	3.5	40	0/6
	2/23/51	17.6	200	0/6
	6/ 6/51		200	2/6
	6/ 8/51		40	2/6
	11/ 8/51		200	3/6
	11/13/51		200	1/6
	11/15/51		200	1/6

*Crude toxin equivalent.

TABLE III. GRADED DOSAGE-RESPONSE OF MONKEYS TO ENTEROTOXIN

SAMPLE NO.	DOSE		NO. POS./NO. FED	
	MG.	ML.*	NEW ANIMALS	USED ANIMALS
1	3.34	40	5/5	1/7
	0.66	8	4/5	2/7
	0.13	1.6	2/5	0/1
	0.027	0.32	0/5	0/1
2	1.5625	40	4/4	2/2
	0.3125	8	2/2	4/4
	0.0625	1.6	4/6	
	0.0125	0.32	3/5	0/1
	0.0025	0.064		0/6

*Crude toxin equivalent.

cases at first suggested the possibility of seasonal variation in susceptibility; however, differences of this magnitude have occurred during short time intervals and in all seasons.

Graded Dosage-Response.—Monkeys which have become resistant to enterotoxin produced by strain 196E appear to be susceptible to toxin produced by strain S-6. Graded dosage-response of such 196E-resistant animals to S-6 enterotoxin is presented in Table III. Animals labeled "new" had never received S-6 toxin; those labeled "used" had previously been fed S-6 toxin one to three times. Toxin sample 1 was lyophilized material concentrated by ammonium sulfate precipitation from a pool of 10-liter cultures produced in turning bottles. Sample 2 was concentrated from 1-liter deep aerated twenty-four hour culture by acid precipitation (production and concentration methods described in previous reports[5, 6]) and was fed before dialysis and lyophilization; the weights given in Table III for this sample were calculated from the nondialyzable material recovered from the remainder of the sample.

The response obtained with toxin sample No. 1 indicates that animals previously fed this toxin were less susceptible than the others. Toxin sample No. 2 was apparently more potent than sample No. 1. In this case the previously used animals were susceptible to large doses, but comparative data were not obtained at lower dosage levels where any acquired resistance should be more apparent.

Although results of this nature (obtained on several occasions) may suggest that perhaps acquired resistance to one strain may not protect against another, we have not at the present time satisfactory data to indicate whether they are qualitatively different enterotoxins. The reverse procedure, i.e., feeding 196E toxin to S-6-resistant animals, has not been done. The distinct impression has been gained that (1) enterotoxin produced by strain S-6 is much more potent than that produced by strain 196E, and (2) monkeys become resistant to S-6 toxin very rapidly, sometimes after only one oral dose. In order to determine whether these differences in toxin potency are qualitative or merely quantitative, special experiments are needed utilizing large numbers of normal animals and designed specifically to resolve this point. The results of such experiments should be much more susceptible to valid statistical

analysis than any data available at present. Such titrations have not been attempted since the great expense of animals involved would prohibit practical utilization of the results in hastening the purification work.

Keeping in mind the above limitations a median-effective dose of 0.219 mg. of toxin No. 1 for "new" monkeys may be calculated by the moving average[16] method.* An interpolated median-effective dose cannot be calculated for sample 2 in "new" animals since the dosage range covered was not low enough. However, it may be assumed that the median-effective dose is 0.0214 mg. or less (all animals used in calculation). The degree of reproducibility of 50 per cent end points using similar 196E-resistant animals or using entirely new animals is not known.

Several factors inherent in the data limit the value of the above calculated median-effective doses of enterotoxin.[16, 17] The number of animals needed in each group for statistically valid results is not known. The feeding tests in this case were not all done at the same time. All animals in a group, regardless of weight, received the same dose of toxin. The animals used cannot be considered normal or selected at random. If S-6 is a qualitatively different toxin, previous feeding of other toxins may have no effect on these tests; if not, all of the animals may have acquired some resistance.

Vomiting by Normal Animals.—Vomiting by healthy animals apparently occurs very rarely. During four and one-half years (with a turnover of approximately 1,000 monkeys) there were recorded 14 instances of vomiting by monkeys not fed enterotoxin or other emetics. Two of these observations involved the same animal. In 6 other instances the animal involved had been fed enterotoxin one to three days previously, and in at least 2 others the animal was obviously ill (diarrhea, emaciation, poor appetite).

There were also recorded 18 instances of unprovoked retching (in 9 of these, stomach contents were regurgitated into the pouches). Seven of these had received enterotoxin one to three days previously and several were obviously ill.

Since there has been relatively little continuous observation of apparently normal animals, the actual incidence of vomiting is not known. Although such unprovoked emesis is probably very rare, it is obvious that the acceptance of a single positive reaction as an indicator of enterotoxin may occasionally be in error. The above observations emphasize the need for selection of only apparently healthy animals for experimental use; also feeding tests should be followed by a sufficient time interval for complete recovery before re-use of animals.

False Positive Reactions.—In preliminary investigations of the solubility of enterotoxin (crude culture supernatant fluid after concentration in vacuo and dialysis) in alcohol-water mixtures, emesis apparently caused by an agent other than the enterotoxin was encountered. Although the enterotoxin was recovered in precipitates, the undialyzed supernatant fluids after removal of alcohol (in vacuo) provoked vomiting when fed to monkeys. The emetic agent

*Toxin No. 1, when fed at a higher dosage (16.72 mg. = 200 ml. crude), caused vomiting in 16 of 18 "new" animals.

in contrast to the enterotoxin, passed through Visking casing. Emesis produced by comparable doses (original volume equivalents) of undialyzed and dialyzed fractions were 6/7 and 0/6, respectively. Similar results with alcohol supernates were obtained using nonenterotoxic cultures (2/2 and 0/2), uninoculated medium (1/1 and 0/2), and amino acid mixtures (5/6). Comparable doses of uninoculated medium and amino acid mixtures (glutamic and aspartic acids in relative concentrations present in the "amigen" medium) not treated with alcohol did not cause vomiting. The agent responsible for these vomiting reactions were not investigated further; salts resulting from pH adjustments may have been responsible.

Intravenous Response.—Vomiting reactions of a monkey undergoing intravenous immunization with a partially purified enterotoxin sample (feeding results 5/6 at 0.9 mg. dosage) are presented in Table IV. This was the toxin sample referred to as "purified" in a previous report.[7] The animal vomited after the first three doses of 0.1 mg., but later tolerated 50 times this dose. These data indicating considerable acquired resistance by the intravenous route are open to the criticism that the sample was not pure, and emetic stimulation by antigenic impurities is a possibility.

TABLE IV. VOMITING REACTIONS CAUSED BY INTRAVENOUS IMMUNIZATION OF MONKEY WITH PURIFIED ENTEROTOXIN

DAY	DOSE-MG.	VOMITING TIMES (MIN.)
1	0.1	35
17	0.1	37, 76, 231
23	0.1	70, 81
32	0.1	
52	0.1	
83	0.5	
88	1.0	
94	2.0	
104	4.0	
110	5.0	

DISCUSSION

It is obvious that the monkey feeding test for staphylococcus enterotoxin (which we consider the most reliable method other than feeding of human volunteers) is far from being a satisfactory assay method.

With regard to the practical problem of determining enterotoxigenicity of staphylococci isolated from foods, some specific recommendations regarding the use of monkeys can be made on the basis of the present work: (1) emesis provoked in at least two different monkeys by oral administration of culture supernatant fluid (or filtrate) indicates that the strain produces enterotoxin. In rare instances the acceptance of one positive reaction may be in error due to vomiting from other causes. (2) To obtain maximum use of animals, samples may be fed first to used animals. If positive results are obtained they are reliable, if the test is negative it should then be repeated with new animals.

False positive reactions such as encountered in our preliminary solubility studies are a special case and merely emphasize the need for controls in experimental work.

Whether monkeys or cats are used in the assay procedure, the general practice at the present time is to prepare test material in semisolid media. Culture in liquid medium in shallow layers[5] yields potent enterotoxin and is a much simpler procedure. However, although good enterotoxin production has been obtained with the few strains tested, the assumption cannot be made that semisolid culture may not be superior for some strains. Comparative results obtained by the two methods with many strains would be useful.

SUMMARY

A wide variation in susceptibility was found in approximately 1,000 *Macaca mulatta* injected intragastrically with staphylococcal enterotoxin. The relative susceptibility of animals can be predicted to some degree on the basis of past response to enterotoxin.

Graded dosage-response curves are not very satisfactory unless new animals are used.

Unprovoked emesis has been observed on rare occasions.

False positive vomiting reactions due to a dialyzable agent have been encountered in experimental preparations. These reactions were promptly detected by the use of proper controls.

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LABORATORY METHODS

AN IMPROVED BLOOD VOLUME METHOD (EVANS BLUE DYE) UTILIZABLE EVEN IN THE PRESENCE OF HEMOLYSIS AND/OR LIPEMIA

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THE clinical value of blood volume determinations has been well established. There is still considerable controversy, however, regarding the accuracy of various methods proposed for such determinations. In general, methods for determining blood (or plasma) volume may be divided into two groups: (1) labeled red cell methods, and (2) plasma diluent methods. The former group includes procedures utilizing carbon monoxide and radioactive isotopes as labeling media. The latter includes procedures in which labeled plasma, or an easily identifiable dye which combines preferentially with plasma protein, is injected.

There has been a lack of agreement between the results obtained by the two types of procedures. However, the viewpoint which is gaining favor is that these methods are complementary.

The purpose of this paper is to present a method for the determination of plasma volume by means of a plasma diluent (Evans Blue dye) which offers the following advantages over the previous procedures of its type: (a) It does not require the use of expensive specialized equipment or the services of highly trained technicians. (b) It utilizes only a single blood sample. (c) It allows blood volume determinations to be made quickly and accurately even in the presence of considerable hemolysis and/or turbidity of the sample.

EXPERIMENTAL DERIVATION OF THE FORMULAS

An examination of Fig. 1 reveals the basis for the procedure described here. The absorption curve of the dye, T-1824,* has a rather broad peak at 600 to 620 millimicrons. Oxyhemoglobin has an absorption curve with characteristic twin maxima at 543 and 578 millimicrons, with minima at 503, 560, and above 610 millimicrons. There is also a maxima below 430 millimicrons. The absorption curve of reduced hemoglobin exhibits the characteristic maxima at 545 millimicrons, and minima above 650 and at 505 millimicrons. There is, in addition, an increase in absorption to a maxima below 430 millimicrons. Both turbid and nonturbid plasma fall steadily in absorption as the wave length increases. However, the rate of fall of absorption of turbid plasma is much slower than that of nonturbid plasma.

It was decided to determine the absorption of both oxidized and reduced hemoglobin at a wave length of 440 millimicrons. At this wave length the relative absorption of the two hemoglobins bears about the same relationship as at 620 millimicrons. Moreover, the

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absorption of T-1824 (Evans Blue dye) at 440 millimicrons is small enough, when compared with the absorption of hemoglobin, to allow an integral blank to be used. A 50 per cent variation in dye concentration at 620 millimicrons results in less than 0.5 per cent variation in apparent hemoglobin concentration at 440 millimicrons. Thus, the maximum error resulting from the assumption of a constant for the dye absorption at 440 millimicrons would be less than 0.25 per cent plus or minus. We used a dye concentration of 1:3,000 as the constant value. This corresponds to a mean plasma volume of 3 liters when the quantity of dye in the procedure described here is used.

The absorption of Evans Blue dye was determined at 620 millimicrons. This is slightly on the long side of the absorption peak in order to minimize the effect of hemolysis. Turbidic light scattering was determined at 710 millimicrons. At this wave length the absorption of all other factors is less than 11 per cent of the turbidic scattering.

The absorption relationships of the various plasma components (oxy- and reduced hemoglobin, Evans Blue dye, and turbidity) were ascertained by a statistical evaluation of the absorption curves for each of the components at the three wave lengths indicated. This was followed by an algebraic equation of the mathematical formulas for the curves obtained. It was felt that this procedure would result in a more accurate evaluation of the relationship than if empirical ratios were used.

First, the mathematical formulas best approximating the absorption curves of oxyhemoglobin at 440 millimicrons and at 620 millimicrons were established. This was done by the following statistical curve-fitting method. Equal increments of an oxyhemoglobin solution replaced like increments of plasma in combination with a saline solution of T-1824 (1:3,000). The absorption of each of these increments was determined using a saline blank. In this way the constant for the plasma absorption was included in the absorption curve for oxyhemoglobin at 440 millimicrons. The absorption of the dye, T-1824, at 620 millimicrons was eliminated by the use of a suitable blank (a 1:3,000 dilution of dye in saline combination), followed by subtraction of the dye constant thus obtained. The results of the experiment are tabulated (Table I).

The absorption curve of oxyhemoglobin at 440 millimicrons followed a nonlinear path while that at 620 millimicrons was approximately linear. The curve at 440 millimicrons appeared to have parabolic characteristics; therefore, curve fitting was attempted on a parabolic pattern. It was first assumed that there would be a uniform statistical distribution about the mean of the increments of hemoglobin used. For simplification of the mathematical evaluation procedure the base of the incremental increases appearing in Table I was changed so that increment No. 3 in Table I corresponded to increment No. 0 in Table II. The normal formula for a parabola, $Y = A + BX + CX^2$, was then evaluated in terms of an arbitrarily chosen parameter, t, corresponding to a unit incremental increase. The formula for the parabolic curve then became: $Y = A + Bt + Ct^2$, where Y is the absorption of the hemoglobin in density units at 440 millimicrons, and A, B, and C are constants characteristic of the curve. The values of the constants were determined by evaluating the first, second, and third moments about the statistical mean of the increments used in terms of the experimental values obtained. This was followed by simultaneous solution of the three equations. The actual data and mathematical evaluation follow.

TABLE I. ABSORPTION OF DYE-PLASMA-SALINE-HEMOGLOBIN AT 440 MILLIMICRONS AND AT 620 MILLIMICRONS

INCREMENT NUMBER	MILLILITERS PLASMA	MILLILITERS HEMOGLOBIN	ABSORPTION IN DENSITY UNITS:		
			440 mμ	620 mμ	620 mμ (CORRECTED)
0	2.00	0.00	0.632	0.271	0.081
1	1.90	0.10	0.927	0.281	0.091
2	1.80	0.20	1.142	0.290	0.100
3	1.70	0.30	1.302	0.300	0.110
4	1.60	0.40	1.402	0.306	0.116
5	1.50	0.50	1.492	0.310	0.120
6	1.40	0.60	1.562	0.318	0.128
Dye blank			0.042	0.190	

The formulas for the moments about the mean of a normally distributed parabolic curve in terms of constants and of the parameter, t, are:

$$m_1 : \Sigma Y = nA + B\Sigma t + C\Sigma t^2$$
$$m_2 : \Sigma tY = A\Sigma t + B\Sigma t^2 + C\Sigma t^3$$
$$m_3 : \Sigma t^2Y = A\Sigma t^2 + B\Sigma t^3 + C\Sigma t^4$$

Since the increments are equal, all terms containing t to an odd power are eliminated. The equations therefore become:

$$(1) \quad Y = nA + C\Sigma t^2$$
$$(2) \quad tY = B\Sigma t^2$$
$$(3) \quad t^2Y = A\Sigma t^2 + C\Sigma t^4$$

As can be seen, (2) yields the value of B directly while the simultaneous solution of (1) and (3) gives A and C. The actual process based on the experimental data is given (Table II).

TABLE II. COMPARISON OF DETERMINED AND CALCULATED ABSORPTION OF ADDED INCREMENTS OF HEMOGLOBIN AT 440 MILLIMICRONS

DETERMINED ABSORPTION AT 440 Mμ	CALCULATED ABSORPTION AT 440 Mμ	ERROR FUNCTION $r_1(Y_c-Y_d)$	$r^2 \times 10^{-6}$	t	t^2	t^4	tY_d	t^2Y_d
0.632	0.650	+0.018	324	−3	9	81	−1.896	5.688
0.927	0.910	−0.017	289	−2	4	16	−1.854	3.708
1.142	1.127	−0.015	225	−1	1	1	−1.142	1.142
1.302	1.298	−0.004	16	0	0	0		
1.402	1.425	+0.023	529	1	1	1	1.402	1.402
1.492	1.508	+0.016	256	2	4	16	2.984	5.968
1.562	1.546	−0.016	256	3	9	81	4.688	14.058
8.459			1895		28	196	4.190	31.966

Solving simultaneously: $A = 1.298$
$B = 0.1497$
$C = -0.02226$

The equation for the parabolic curve representing the absorption of hemoglobin at 440 millimicrons therefore becomes:

$$Y = 1.298 + 0.1497t - 0.02226t^2$$

The standard error of estimate resulting from the use of the above equation is 1.13 per cent.

Since the absorption curve at 620 millimicrons was approximately linear, it was not necessary to use as complicated a curve-fitting procedure as the above. In this instance, the general equation for a line in terms of an unknown, X, corresponding to the absorption of hemoglobin at 620 millimicrons, was employed. As before, a parameter, t, corresponding to a unit incremental increase, was used. The results of the mathematical evaluations of the hemoglobin absorption curve at 620 millimicrons are tabulated (Table III).

Let $X = \bar{m}t + C$ where $\bar{m} = \Sigma(X_{i+1} - X)/n$ and $C = X$

$\bar{X} = 0.7670/7 = 0.1096 = C$

$\bar{m} = 0.0560/6 = 0.0093$

Therefore, $X = 0.0093t + 0.1096$

The standard error of estimate of this equation is 0.48 per cent.

The two curves obtained above (that at 440 millimicrons and that at 620 millimicrons) were then equated in terms of the common parameter, t.

Therefore: $Y = -3.557 + 72.52X - 257.25X^2$

or

$$(I) \quad X = 0.1409 - 0.06236\sqrt{1.543 - Y}$$

In order to obtain a greater degree of precision and to enable the procedure to be applied to turbid plasma samples, the following formula was derived. Curves corresponding to the light scattering due to turbidity were obtained in a manner similar to that used for hemoglobin absorption. In this case, increments of nonturbid, nonhemolyzed plasma were replaced with equal increments of turbid nonhemolyzed plasma. The resulting equations are:

TABLE III. COMPARSION OF DETERMINED AND CALCULATED ABSORPTION OF ADDED INCREMENTS OF HEMOGLOBIN AT 620 Mμ

ABSORPTION OF HEMOGLOBIN AT 620 Mμ DETERMINED (X_d)	ABSORPTION OF HEMOGLOBIN AT 620 Mμ CALCULATED (X_c)	$r_i (X_c - X_d)$	MEAN INCREMENTAL INCREASE (M)	r_i^2 x10^{-8}	$\bar{m}t$
0.0810	0.0817	+0.0007	0.010	49	−0.0270
0.0910	0.0910	0.0000	0.009	0	−0.0186
0.1000	0.1003	+0.0003	0.010	9	−0.0093
0.1100	0.1096	−0.0004	0.010	16	0.0000
0.1190	0.1189	−0.0001	0.009	1	+0.0093
0.1290	0.1282	−0.0008	0.008	64	+0.0186
0.1370	0.1375	+0.0005		25	+0.0277
0.7670			0.056		

(II) At 440 millimicrons in terms of the light scattering, Z, observed at 710 millimicrons:

$$Y = Y^o - 0.473Z + 0.0086$$

(III) At 620 millimicrons in terms of the light-scattering, Z, observed at 710 millimicrons:

$$X = X^o + 1.050Z - 0.381$$

The standard error of estimate of this equation if 0.835 per cent. Combining equations (II) and (III) with the basic equation (I) obtained above:

$$(IV)\quad X = 1.050Z + 0.0647 - 0.06236\sqrt{1.543 - Y + 0.473Z - 0.0086}$$

The standard error of estimate of the completed equation is approximately 1.65 per cent.

This formula was applied to a number of human plasma samples exhibiting varying degrees of turbidity and hemolysis to which were added known amounts of T-1824 (Evans Blue dye). In each sample, 2.0 ml. of plasma was added to 4.0 ml. of normal saline containing a known amount of dye. The dye concentration was arranged to approximate that

TABLE IV. COMPARSION OF CALCULATED AND DETERMINED DYE CONCENTRATIONS

SAMPLE NUMBER	CONCENTRATION OF DYE PRESENT × 10^{-4}	CONCENTRATION OF DYE DETERMINED × 10^{-4}	ERROR $r_i = C\text{-}C_o$ × 10^{-4}	PER CENT ERROR RESULTING FROM DETERMINATION
22-3	3.333	3.350	0.027	0.666
23-1	3.333	3.290	−0.043	−1.332
24-3	3.333	3.390	0.057	1.859
27-3	3.910	3.985	0.075	1.938
28-3	3.910	3.860	−0.050	−1.326
29-3	3.910	4.070	0.080	2.063
30-3	3.910	3.940	0.030	0.714
38-3	2.478	2.480	0.002	0.008
39-3	2.478	2.506	0.028	1.130
40-3	2.478	2.496	0.018	0.726
41-3	2.478	2.440	−0.038	−0.534
01-4	3.598	3.650	0.052	1.445
02-4	3.598	3.566	−0.032	−0.879
14-4	3.598	3.550	−0.048	−1.335
15-4	3.598	3.636	0.038	1.055
16-4	3.598	3.518	−0.080	−2.225
17-4	3.640	3.646	0.006	0.016
18-4	3.640	3.660	0.020	0.049
19-4	3.640	3.570	−0.070	−1.870
20-4	3.786	3.820	0.034	0.898
21-4	3.786	3.826	0.40	1.000
22-4	3.786	3.730	−0.056	−1.378
23-4	3.786	3.746	−0.040	−1.000
24-4	3.786	3.704	−0.082	−2.218
25-4	3.786	3.806	0.020	0.529

The average error resulting from the above experimental dilutions as compared with the determined dilutions is *1.208 per cent.*

DENSITY UNITS

which would be obtained from the injection of 3.0 ml. of dye (0.5 per cent solution) into a plasma volume of 3 liters (Table IV). The average error resulting from the application of the equation (IV) to the experimental dilutions was 1.208 per cent.

METHOD

Exactly 3 ml. of a 0.5 per cent solution of Evans Blue dye is injected into either the right or left antecubital vein. In order to allow an exact volume of dye to be injected, a special "Chaney" syringe* fitted with a syringe valve is used. The dye solution is introduced slowly into the vein over a period of approximately twenty seconds. Ten minutes later 11 ml.

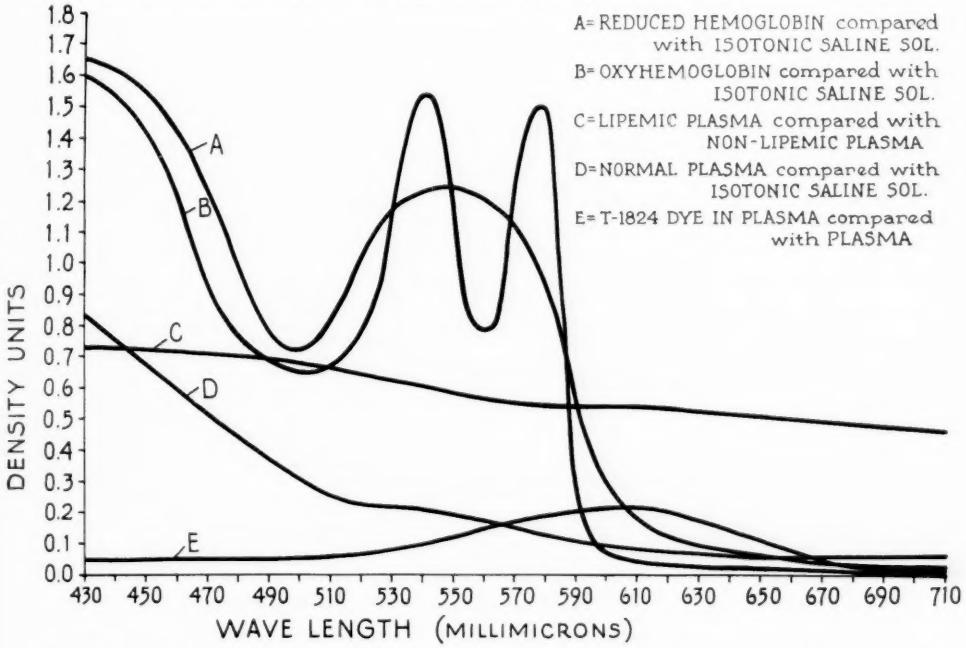

Fig. 1.—Spectrophotometric absorption curves of reduced hemoglobin, oxyhemoglobin, normal, lipemic, and dyed plasmas.

of blood is withdrawn from the opposite antecubital vein with a syringe containing not more than 0.1 ml. of heparin solution. Care must be taken to avoid stasis of the circulation when this sample is obtained. For this reason, a tourniquet is not used and the sample is withdrawn slowly.

The sample is run into a 12 ml. conical centrifuge tube containing 5 mg. of potassium-ammonium oxalate powder. After thorough mixing of the blood with the powder, 1 ml. of the sample is transferred to a Wintrobe hematocrit tube. Both tubes are then centrifuged at a speed producing a maximum R.C.F. of 2,080g at the tip for a period of 30 minutes. A hematocrit correction factor of 0.915, as recommended by Chapin and Ross,[1] is applied to allow for erythrocytic trapping of plasma.

Two milliliters of the plasma is transferred to a cuvette, diluted with 4 ml. of normal saline solution, and read against a normal saline solution blank in a Coleman spectrophotometer (model No. 14) or in an instrument having an equivalent band width and resolution at each of the following wave lengths: 440 millimicrons, 620 millimicrons and 710 millimicrons. A standard dye solution (a 1:2,000 dilution of a 0.5 per cent solution of Evans Blue dye in normal saline, freshly prepared) is used as a standard and to check the calibration of the instrument. Two milliliters of normal plasma is added to 4 ml. of the dye solution and read at the above three wave lengths against a dilution of the same plasma with undyed normal saline solution.

*Microchemical Specialties, Berkeley, Calif.

A formula is applied to the readings obtained at wave lengths of 440 millimicrons, and 710 millimicrons. This yields a blank which when subtracted from the reading at wave length, 620 millimicrons, gives the dye absorption at the last frequency. The entire mathematical calculation is as follows:

Let Y = observed absorption in density units at 440 mμ
Let X = observed absorption in density units at 620 mμ
Let Z = observed absorption in density units at 710 mμ
Let D_u = actual absorption in density units of the dye in the sample at 620 mμ
Then:

$$D_u = X - (1.050Z + 0.0647 - 0.06236 \sqrt{1.543 - Y + 0.473Z - 0.0086})$$

The standard error of estimate of this equation is 1.65 per cent.
Therefore: Plasma volume = $D_s/D_u \times 3000$
Where D_s is the absorption in density units of a 1:3,000 dilution of Evans Blue dye at 620 millimicrons:

Blood volume = Plasma volume × 1/1 − corrected hematocrit.

RESULTS

In 19 normal women the mean plasma volume was 1.589 liters per square meter of body surface, with a standard deviation of ± 0.183 liter per square meter. The mean blood volume was 2.475 liters per square meter of body surface, with a standard deviation of ± 0.178 liter per square meter. In 30 normal men the mean plasma volume was 1.740 liters per square meter of body surface, with a standard deviation of ± 0.291 liter per square meter. The mean blood volume was 2.910 liters per square meter of body surface, with a standard deviation of ± 0.411 liter per square meter.

The Evans Blue dye method yields higher values for the plasma volume and for the total blood volume than do methods employing labeled erythrocytes. This difference may be due to dye diffusion into those portions of the noncirculating plasma where erythrocytes are unable to enter.[2]

DISCUSSION

When Evans Blue dye (T-1824) is injected in massive doses into the blood stream, there occurs staining of the endothelial cells and other parenchymal tissues. However, in amounts required for blood volume determination, even when repeatedly injected, the dye does not appear to be retained by endothelial or other tissues.[3] It has been demonstrated that T-1824 is completely combined with albumin when in a relative concentration of 0.006 mole per cent or less.[4] Thus, the usual amount of dye injected for the determination of the blood volume (15 mg.) would be bound by the albumin contained in approximately 67 ml. of plasma. The combination of the dye with albumin is sufficiently rapid to permit binding of the dye in the time necessary for one complete circulation of the blood.[5] The dye-albumin combination is very stable and has a free energy charge of combination of 8,750 calories per mole of dye.[6] The problem is to prevent binding of the dye with the protein of the endothelium or other body tissues. This can be accomplished either by injecting a dye-albumin combination or by injecting the free dye over a period long enough to permit complete combination with the serum albumin to occur at a point close to that of entrance. The latter technique was used here.

Three methods have been employed for the spectrophotometric evaluation of T-1824 concentration in plasma or serum. Gibson and Evans[7] developed a formula based on the ratio of dye absorption at 574 millimicrons to that at 620 millimicrons. Their formula suffers from three defects. First, because hemoglobin absorption is measured at a point at which dye absorption is quite high, it is necessary to have at least one of the samples nonhemolyzed. Second, the formula is not valid when appreciable amounts of reduced hemoglobin are present due to the difference in absorption of oxyhemoglobin and reduced hemoglobin at 574 millimicrons. Third, the formula is not valid in the presence of turbidity of the blood sample. The formula of Gaebler[8] is based on serum absorption at 577 millimicrons, at 630 millimicrons, and at 750 millimicrons. This formula is limited in usefulness by the same factors which affect the formula of Gibson and Evans. In addition, the turbidic measurements are inconsistent at the deep red wave length which is used. There does not seem to be adequate justification for determining dye concentration at a point on the slope of the curve rather than at the peak. A formula has been proposed by Shapiro[9] which is based on the determination of the dye absorption at 620 millimicrons and at 680 millimicrons compared with an integral blank. We were unable to confirm the validity of this formula when hemolyzed plasma was used. Recently, a procedure has been devised by Allen[10] in which the dye, T-1824, is extracted when gross hemolysis or lipemia is present in the blood sample. Allen's method requires two blood samples and a separate extraction procedure.

SUMMARY

A simple and accurate Evans Blue dye method for the determination of the blood and plasma volumes is described. The procedure can be used even in the presence of lipemia and/or hemolysis. The blood volume measurements obtained by the application of this method to 49 men and women are presented.

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PHOTOGRAPHIC METHOD FOR ENUMERATION OF ASHBY AND TOTAL RED CELL COUNTS*

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WHEN performing Ashby counts there are certain advantages of counting from photographs over direct cell counting of hemocytometer spreads of red cells through the microscope.

Advantages.—

1. Technicians can prepare larger numbers of counts in one laboratory session than is possible if the counts are made directly at the time of their preparation.

2. The photographs can be made rapidly without fear of evaporation from the chambers which might occur otherwise during direct counts of large numbers of cells.

3. The actual counting from films can be done in relative comfort with less eyestrain and fatigue.

4. While there is probably no actual saving of counting time, the technicians can apportion their time better and enumerate the counts when most convenient.

5. The films permit a permanent record of each count made.

Disadvantage.—

1. The counts cannot be checked until the film has been developed, fixed, washed, and dried. Even though a number of films may be processed simultaneously, this procedure requires approximately forty-five minutes to one hour.

MATERIALS AND METHODS

After trials with numerous types of cameras, photomicrography equipment, films, and micro-readers, the following equipment was selected as the most economical and suitable for this purpose:

Camera.—We employ a Visicam microscope camera with Argus 35 mm. back.† This simple camera contains no lenses, and fits over the ocular of any monocular microscope (Fig. 1). The only focusing adjustments necessary are made in the conventional way on the microscope. A ground glass viewer with a central clear area for fine focus allows visual alignment and sharp focus of the image in a magnification up to the width of the film frame.

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*The idea of using photographs for enumerating Ashby counts was originally suggested to us by Dr. William Thalhimer of Valhalla, N. Y. Recently, Dr. Thalhimer stated in a personal communication to the author that since making this suggestion to us he has also developed a successful photographic technique for enumerating Ashby counts. The use of photographs for enumerating total red cell counts has been described previously.[1]

†Both obtainable from Erb and Gray Company, Los Angeles, Calif.

Light Source, Light Filter, and Base.—In order to achieve maximum sharpness and detail, a powerful, properly focused light source was found absolutely essential. Once such a light source was aligned and focused, the alignment was maintained by employing a base* to which both the microscope and lamp could be clamped in permanent position (Fig. 1). We employ a microscope lamp† with a ribbon filament bulb controlled by a three step transformer (Fig. 1). In order to avoid artifacts on the films caused by dust or bubbles in the lamp water cell holder, this cell is filled with clear silicone fluid.‡ We use a Wratten No. 67 blue green filter§ in the lamp's filter holder to increase contrast of the red cells against the background. A wide, camel's-hair brush is useful to keep the filter surfaces free of dust.

Illumination.—For those lacking experience in photomicrography, the essentials of the Kohler illumination technique[2] are as follows: the light source is placed at that distance from the microscope where the sharpest focus of the rectangular light beam fills the underside of the closed substage iris diaphragm when the latter is viewed in the plain substage mirror.

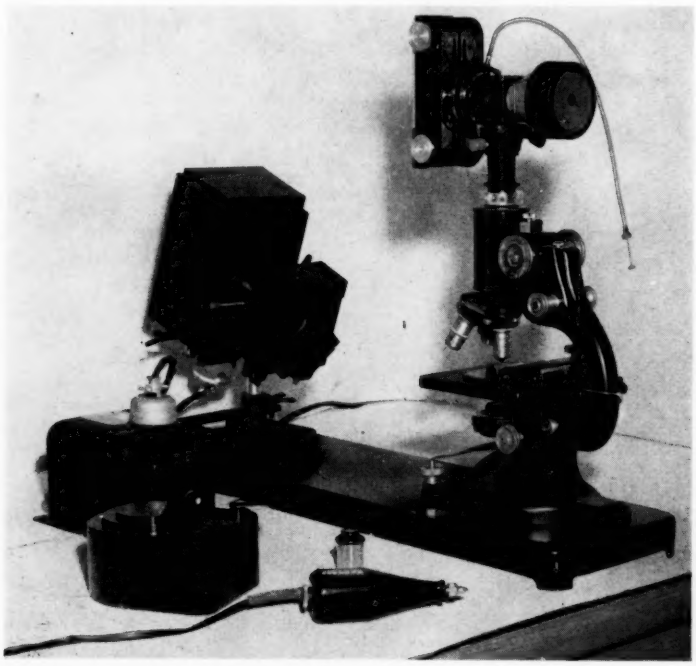

Fig. 1.—Equipment used for photographing spreads of red cells in hemocytometers. Note the lamp and microscope are permanently locked into position by the base. Lamp transformer, daylight film loader, 10× ocular, and film marking tool are shown in left foreground.

This is accomplished by proper positioning of the lamp, and by simultaneously focusing the lamp lenses. Both the lamp and its lens system should be locked permanently in place when this focusing adjustment is completed. Once the light source is positioned and lamp lenses properly focused, the light is centered in the ground glass viewer of the camera with the substage iris diaphragm open. The light centering should be tested by closing down the iris diaphragm on the lamp. The resulting small circle of light should coincide with the central clear focusing circle of the camera viewer. With ordinary microscopes, the light may be centered by adjusting the plain substage mirror. The completely adjustable substage should be used for centering the light on research microscopes.

*Obtainable from Bausch and Lomb Company, Rochester, N. Y.
†Bausch and Lomb Research Microscope Lamp, Type 31-33-85.
‡D C 200 fluid, 1,000 centistoke viscosity, obtainable from Dow-Corning, Midland, Mich.
§Obtainable from Eastman Kodak Company, Rochester, N. Y.

The final focusing of the light source is made with the ruled field of a filled counting chamber in focus under the low power (10×) objective. With the field in good focus on the ground glass viewer, a pointed object (sharp pencil) should be placed against the front of the light source and the substage condenser moved slowly up or down until the shadow image of the pencil point is brought into sharp focus on the viewer. The substage condenser should remain at this position. This latter test of focus of light source should be checked before each day's photographing.

For maximum sharpness and depth of field, the light in the ground glass viewer should be diminished by closing the substage iris diaphragm until just before a circular shadow begins to creep inward from the periphery of the viewer.

The optimum substage iris diaphragm setting (which may be left constant once it has been obtained) should be determined by a few trial photographs; it will vary with the film and shutter speeds. Practically all of the focusing adjustments mentioned above need be made only initially as long as the equipment is left undisturbed when not in use. A fine focus check of the microscopic field is made through the central clear spot of the viewer with a 10× ocular before each photograph.

Film.—A Bantam sized camera back is also obtainable* for the Visicam Camera (this allows some increase in field magnification), but we have preferred to use 35 mm. film which

TABLE I. COMPARISON OF RED CELL COUNTS OBTAINED BY ENUMERATING DIRECTLY THROUGH THE MICROSCOPE AND FROM PHOTOGRAPHS OF THE SAME SPREADS

COUNT NUMBER	CHAMBER NUMBER	CHAMBER AREA COUNTED (SQ. MM.)	CELL COUNT DIRECT	CELL COUNT FROM PHOTOGRAPHS	CELLS/MM.^{3} DIRECT COUNT × 10^3	CELLS/MM.^{3} PHOTOGRAPH COUNT × 10^3
*A. Inagglutinable (Ashby) Counts**						
1	1st	5.0	1226	1224	473.	474.
	2nd	5.0	1146	1143		
2	1st	5.0	542	549	213.	211.
	2nd	5.0	514	517		
3	1st	9.0	484	482	109.	109.
	2nd	9.0	494	497		
4	1st	9.0	260	261	59.	59.
	2nd	9.0	267	267		
5	1st	4.0	936	942	466.	463.
	2nd	4.0	913	922		
6	1st	4.0	910	915	238.	237.
	2nd	4.0	984	990		
7	1st	9.0	1475	1480	156.	156.
	2nd	9.0	1324	1327		
8	1st	9.0	975	977	50.	50.
	2nd	9.0	998	1000		
B. Total Red Cell Counts†						
1	1st	0.2	369	368	3570.	3580.
	2nd	0.2	345	348		
2	1st	0.2	407	401	3975.	3985.
	2nd	0.2	388	396		
3	1st	0.2	473	464	4580.	4575.
	2nd	0.2	443	451		
4	1st	0.2	528	524	5180.	5190.
	2nd	0.2	508	514		
5	1st	0.2	519	526	5085.	5110.
	2nd	0.2	498	496		

*A. Eight inagglutinable (Ashby) counts ranging from 50,000 cells per cubic millimeter to 473,000 cells per cubic millimeter.

†B. Five total red cell counts ranging from 3.57 × 10^6 per cubic millimeter to 5.18 × 10^6 per cubic millimeter. The differences between the direct counts and counts from photographs of the same spreads are insignificant. In actual practice, it is best to enumerate larger numbers of inagglutinable cells than listed above, preferably from more than two spreads, in order to decrease the errors (mixing, distribution, pipette, chamber, etc.) inherent in all red cell counting.

*From Erb and Gray Company, Los Angeles, Calif.

can be obtained economically in bulk 100 foot rolls. Eastman Micro File film* is capable of resolving 135 lines per millimeter, and will give the sharpest detail of cells when projected for counting. The film may be loaded into cartridges in any lengths up to 38 frames by any 35 mm. daylight film loader.

Micro File film should be developed in Eastman D-11* high contrast developer for four minutes at 68° C. The exposed end-tab of the film may be marked with the patient's name, date, and other identifying data by scratching the film. We have found a small vibrating marking tool† ideal for this purpose. Each spool of film is marked before insertion into the camera.

By using a monocular microscope equipped with a 6× ocular and adjustable ocular tube and employing the low-power 10× objective, 1 square (1.0 sq. mm.) of the ruled area of

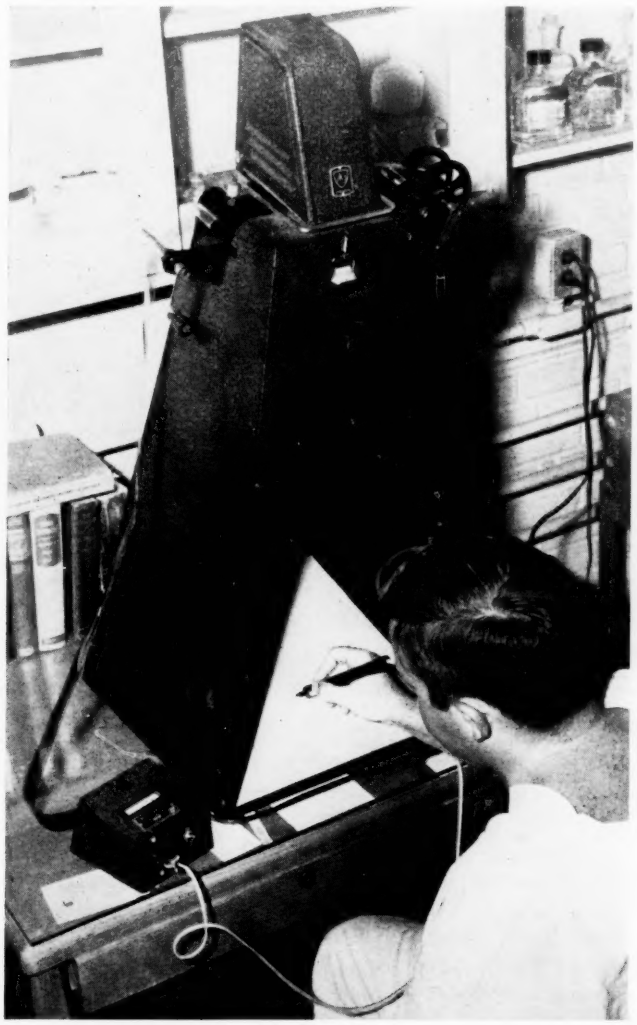

Fig. 2.—Showing micro-reader with film in place. The electric colony counter is shown in use, but for this purpose it has no special advantage over the hand tally counters.

*Obtainable from Eastman Kodak Company, Rochester, N. Y.

†Burgess Vibro-Graver, manufactured by Burgess Battery Company, Handicraft Division, Lake Zurich, Ill.

the counting chamber can be brought to fill the width of the 35 mm. × 24 mm. ruled area of the ground glass viewer. This permits a magnification of the "square" to the full width (24 mm.) of the 35 mm. film.

As a rough exposure guide to others interested in this method, we use a substage iris diaphragm setting of 0.25 and a shutter speed of 0.5 second when employing Micro File film.

Film Projecting and Counting.—In our laboratory, both chambers of the two hemocytometers are filled from four separately prepared serum-cell mixture tubes for each Ashby count. A sufficient number of large squares (1.0 sq. mm.) of each spread (depending on the blood dilution and inagglutinable count) are photographed to enable us to enumerate a total

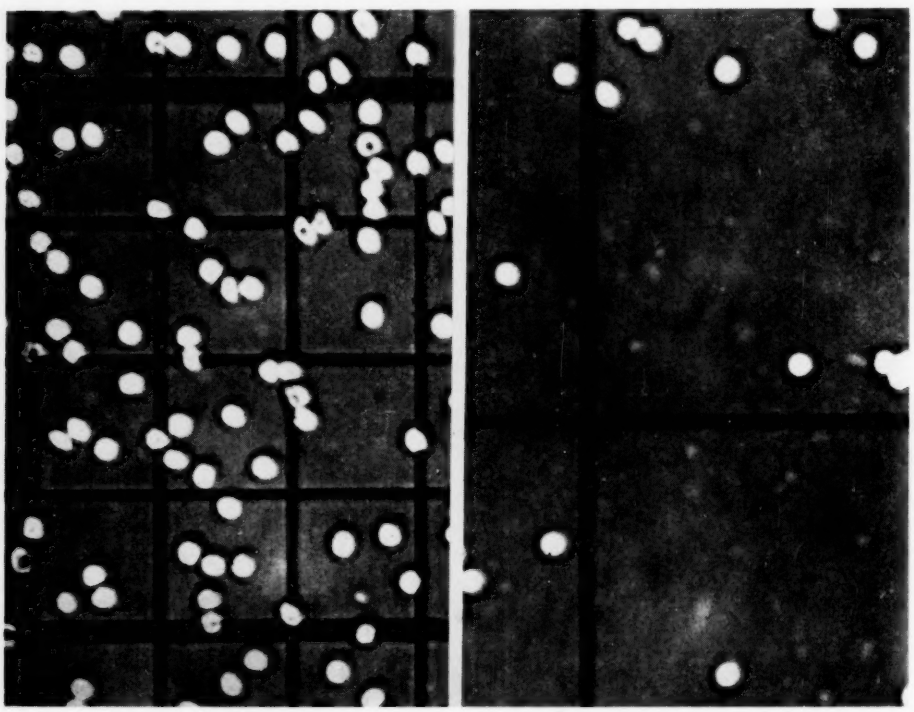

Fig. 3.—*Left:* A portion of a photograph of a center square (1.0 square millimeter) from a total red blood cell count enlarged to the exact size as seen when projected in the micro-reader, for counting. When projected in the micro-reader, the whole square (1.0 square millimeter) would be visible at this magnification. *Right:* A similar magnification of a portion of a photograph taken of a large corner square (1.0 square millimeter) from an inagglutinable (Ashby) count.

of at least 500 and preferably up to 4,000 inagglutinable cells per Ashby count. When determining the recipient's baseline inagglutinable count or when the number of transfused inagglutinable cells have fallen to low levels, we photograph all 36 squares of the 4 spreads (final blood dilution 1/50). With each Ashby count, the center squares of two additional spreads from separate R.B.C. pipettes are photographed for enumerating the total red cell count. Thus, at most, 38 frames are sufficient for any Ashby count including the total red cell count. When 38 frames are needed on one roll, they can be accommodated in one 35 mm. cartridge by loading the film in a darkroom. Thus, when employing potent antisera permitting low dilutions (1/50) of original blood, this maximum of 38 film frames per cartridge supplies sufficient photographs for enumerating 500 free cells even when the number of inagglutinable cells are minimum (6,000 to 8,000 per cubic millimeter).

For counting, we use a regular micro-reader* for 35 mm. film (Fig. 2). This reader projects the photographs (negatives) of each large square to a magnification of 14 inches square which allows clear identity of all unagglutinated red cells (Fig. 3). A projection screen for the base of the reader can be made of clear x-ray film. With such a screen, an ink writing, electric, colony counter,† as illustrated in Fig. 2, may be used to mark off each cell as it is automatically counted. The ink marks from a previous count may be quickly wiped from the x-ray film with a damp cloth before beginning the count of the next square. However, after experience, we found the ordinary hand tally counter to be as convenient and more rapid than the colony counter.

Both Ashby and total red cell counts enumerated directly through the microscope and again later, from photographs of the same original spreads, show insignificant differences resulting from the two methods of counting (Table I).

SUMMARY

Simple photomicrography and projection equipment are described for a convenient method of enumerating Ashby or total red cell counts from photographs of hemocytometer spreads of red cells. No additional source of error is introduced by enumerating free cells from photographs. The various advantages of this method of counting are listed.

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*Spencer 35 mm. Micro-Reader, manufactured by American Optical Company, Buffalo, N. Y.

†Klett Colony Counter obtainable from Klett Manufacturing Company, New York, N. Y.

STUDIES ON PRIMARY AMINES

I. Methods

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MANY pressor principles have been found in blood,[1-6] urine,[7-14] and kidneys[15-17] of hypertensive patients and normal subjects and of experimental hypertensive animals. The vasoconstrictor substance in normal serum has been identified.[18] Holtz[19, 20] showed that histidine, tyrosine, and dihydroxy-phenylalanine give rise to pressor substances under conditions of low oxygen tension in vitro. Presumably, the pressor substances are the corresponding amines. Evidence of this sort was also obtained by Bing and associates[2] and Blaschko.[21]

Final proof that decarboxylation without deamination is involved in human hypertension will require the extraction and chemical identification of the pressor amine(s) from human blood. Evidence in this direction has been obtained,[1, 22] and is now in an advanced stage.[23]

An examination of the literature failed to show that methods to study the occurrence of primary amines in biological fluids were available. Guggenheim[24] has described a number of reactions of the amines. Richter[25] has used the color developed by picric acid as an index for the presence of organic bases which form picrates. Methods for the determination or estimation of histamine,[26] cinchonidine[27] and other bases,[28] hexosamines,[29, 30] arterenol,[31] and the catechol group[32, 33] have been devised. Hydroxytyramine has been isolated from urine,[34] but reports of successful attempts to isolate, identify, and measure primary amines as a group of compounds in biological fluids have not been found. The present report outlines the methods used in the initial stages of a project aimed at discovery of the defects which occur in intermediate metabolism of hypertensive individuals. By the methods described in this paper, as many as six primary amines have been isolated from single twenty-four-hour samples of urine. The methods of separation, partial identification, and quantitation will be discussed. Subsequent papers will describe in greater detail the limiting and optimum factors in the determination, the extraction of amines from biological fluids, and studies on these substances in normal and hypertensive subjects.

METHOD

Reagents:

Ninhydrin reagent. 0.2 per cent ninhydrin in isopropanol.

Acid-isopropanol mixture.

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Dilute one volume of concentrated hydrochloric acid to 100 volumes with isopropanol.

Pyridine, reagent grade.

Stock solutions of amine hydrochlorides. 0.01 molar solutions of amines are made up in acid-isopropanol mixture. Mall all dilutions of stock solutions with acid-isopropanol mixture.

Pyridine-Ninhydrin reagent. Prepare 0.2 per cent ninhydrin in isopropanol containing 20 per cent pyridine.

Three approaches have been made to the separation of amines from other constituents of biologic fluids. Each has its advantages. Extraction of bases into organic solvent from alkaline aqueous solution may be carried out by batch or continuous extraction. Ion exchange

TABLE I. RF VALUES OF SELECTED AMINES ON ASCENDING PAPER CHROMATOGRAM USING BUTANOL-WATER SOLVENT

AMINES*	RF	COLOR†	RF FROM LITERATURE‡	REMARKS
Simple Aliphatic				
Methylamine	0.10	Bl-Pur	0.10	
Ethylamine	0.17	Pur	0.18	
2-Mercaptoethylamine	0.013	Pur	0.52	
n-Butylamine	0.44	Pur	0.31	
3-Methyl butylamine	0.53	Pur	0.52	Syn: isoamylamine
n-Hexylamine	0.66	Pur		
Cyclohexylamine	0.54	Pur-Red		
2-Hydroxy-hexylamine	0.19	Pur		Syn: 1-amino, 2-hexanol
Cystamine-2 HCl	0.11	Pur		
Glucosamine	0.052	Pur	0.04	
Glutamine	0.045	Pur		
n-Heptylamine	0.71	Pur	0.58	
1,3-Dimethyl pentylamine	0.69	Pur		
1-Methyl heptylamine	0.75	Pur		
n-Decylamine	0.75	Pur		
n-Dodecylamine	0.76	Pur		
n-Tetradecylamine	0.80	Pur		
n-Octadecylamine	0.94	Pur		
Aromatic Substituted				
2-Phenethylamine	0.48	Pur	0.51	Syn: phenylethylamine tyramine
2-(4-Hydroxyphenyl) ethylamine	0.33	Br-Red	0.30	
2-Hydroxy, 2-(3,4-dihydroxy phenyl) ethylamine	0.13	Br		Syn: nor-epinephrine; arterenol
2-Hydroxy, 2-(3,4-dimethoxy phenyl ethylamine	0.32	Red-Br		
1-Methyl, 2-hydroxy, 2-phenyl ethylamine	0.54	Pur		Syn: Propadrine
1-Methyl, 2-(4hydroxyphenyl) ethylamine	0.57	Pur		Syn: Paredrine
1-Methyl, 2-(3,4-dihydroxyphenyl ethylamine	0.37	Or		
1-Butyl, 2-hydroxy, 2-phenyl ethylamine	0.74	Pur		
α-Amino, 3,4-dihydroxy acetophenone	0.24	Or		Syn: arterenone
α-Amino, diethylamino, 3,4-dihydroxyacetophenone	0.26	Br-Pur		Very faint color
Heterocyclic				
Histamine	0.061	Red-Br	0.03	
Tryptamine	0.38	Pur-Br	0.35	
Hydroxytryptamine creatinine SO_4	(0.019	Br		Syn: Serotonin
	(0.20	Br		

*All amines were crystalline hydrochlorides or were made up in a hydrochloric acid solvent.

†Bl, Blue; Pur, purple; Br, brown; Or, orange.

‡Reference 36.

resins are effective for amines destroyed at high pH. The present paper concerns only the separation and quantitation of amines after they have been removed from other constituents of biological fluids.

Separation by Paper Chromatography.—One-dimensional ascending paper chromatography using a modification of the Williams and Kirby method[35] was found satisfactory for most purposes. The pyridine-ninhydrin solution is used for spraying the chromatogram. In the absence of pyridine basic amino acids react with ninhydrin in isopropanol, but amines and neutral amino acids develop color slowly. In addition, colors are generally blue without pyridine. With pyridine, colors vary from pink through purple to brown, and chemical characteristics can often be recognized in this way. No advantage was found in two-dimensional chromatograms using water-saturated phenol and butanol. The most useful solvent tested so far is butanol saturated with water. Table I shows the mean Rf values, the color of the ninhydrin complexes of the simple, the aromatic, and heterocyclic substituted aliphatic amines, and comparison with Rf values in the literature. Close agreement with Rf's reported by Bremner and Kenten[36] was obtained. Aliquots of extracts from twenty-four urine samples representing a quarter of the sample are chromatographed on the same paper with selected amines of biological interest. The amines appearing above the extract after spraying are compared with the Rf values, colors, and rates of color development of known amines. When the identification of an unknown amine is sought, it is re-chromatographed on two spots, one strip of which is sprayed. The ninhydrin spot of interest is eluted for spectrophotometric reading; its partner is eluted for further tests.

Positive identification of any compound can never be made by chromatography alone, but the Rf range, the color, and the rate of color development can greatly assist in characterizing the structure of unknown compounds. Hydrophilic compounds tend to have low

TABLE II. RELATIVE DENSITY AT 400 AND 570 Mμ OF SELECTED ALIPHATIC AND AROMATIC-SUBSTITUTED NINHYDRIN-AMINE COMPLEXES ELUTED FROM PAPER CHROMATOGRAMS

AMINE HYDROCHLORIDE	CONCENTRATION IN ELUATE, μM	DENSITY* AT 400 Mμ	DENSITY* AT 570 Mμ	RATIO 400/570 Mμ
	Simple Aliphatic			
Methylamine	500	0.134	0.142	0.944
Ethylamine	333	0.254	0.291	0.873
n-Propylamine	333	0.292	0.323	0.904
n-Butylamine	333	0.349	0.365	0.956
n-Amylamine	333	0.427	0.437	0.977
Isoamylamine	333	0.423	0.434	0.975
	333	0.266	0.280	0.950
	333	0.423	0.453	0.934
n-Hexylamine	500	0.490	0.498	0.984
1-Methyl heptylamine	250	0.106	0.084	1.262
Decylamine	400	0.208	0.207	1.005
	S- and O- Substituted Aliphatic			
Glutamine	1000	0.423	0.468	0.904
β-Mercaptoethylamine	500	0.237	0.172	1.378
Cystamine	333	0.430	0.460	0.935
	Ring-Substituted			
β-Phenylethylamine	2500	0.261	0.174	1.500
Tyramine	1000	0.529	0.387	1.367
Nor-epinephrine	333	0.305	0.204	1.495
Arterenone	333	0.173	0.106	1.632
Tryptamine	400	0.256	0.162	1.580
Serotonin creatine SO_4 (5-OH tryptamine)	166	0.228	0.099	2.303
Histamine	500	0.220	0.147	1.497
dl-p-Hydroxy-α-methyl-phenethylamine	333	0.116	0.096	1.208

*Maximum density readings of both peaks of the ninhyhydrin-amine complexes shift toward the ultra violet region when they are eluted from paper with water. Therefore 400 and 570 rather than 415 and 575 mμ readings are used

Rf values. The colored complex can be eluted with water directly from paper and read in the Beckman spectrophotometer. When this is done there is a shift in the spectral absorption curves from those obtained in the test tube reaction to be discussed. Simple aliphatic and aromatic-substituted aliphatic amines can thus be differentiated by comparison of spectrophotometric readings at 400 and 570 mμ. If the latter is lower than the former the compound usually has a ring substitution (Table II).

The length of the chain of simple, unbranched aliphatic amines bears a direct relation to Rf value. Compounds with less than seven carbon atoms have Rf's which increase by increments of 0.11 per carbon atom. Rf values of amines with chain lengths of seven to 14 carbons increase on an average by an increment of only 0.014 per carbon atom. This is best shown in Fig. 1. One possible explanation would involve the spatial configuration resulting from a tendency toward ring formation at six carbons. Isoamylamine and other branched aliphatic amines fall on the curve roughly according to total number of carbon atoms. Sulfhydryl and hydroxyl substitutions in ethylamine lower the Rf value.

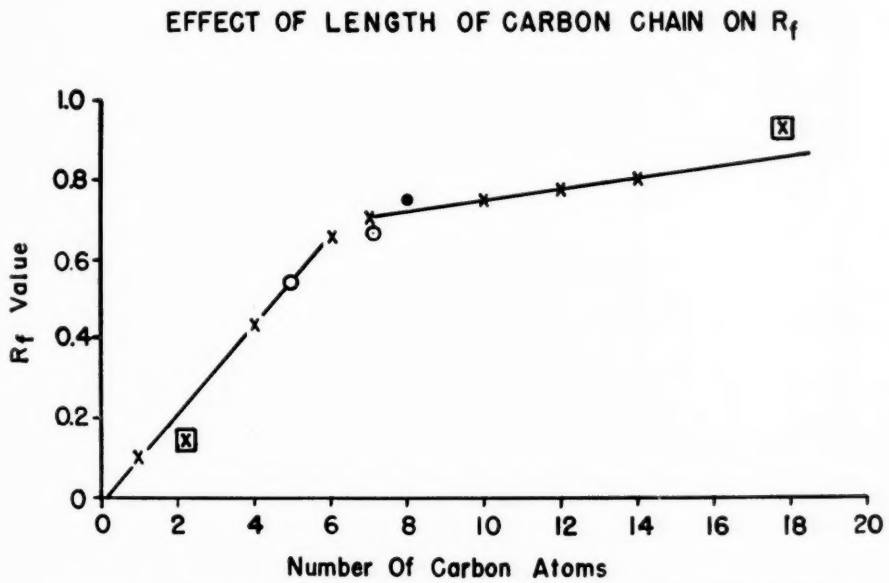

Fig. 1.—Effect of length of carbon chain and number of carbon atoms on Rf values of aliphatic primary amines in butanol–H_2O. *X*, unbranched, saturated primary amines; □, Rf values taken from previous chromatograms; ○, isoamylamine; ⊙ 1,3-dimethylpentylamine; ● 1-methylheptylamine. See text.

The effect of length of side chain and of position and number of hydrophilic groups in the molecule was examined in the aromatic series. From results on the available compounds certain generalizations can be safely made. As in the simple aliphatic series Rf increases with length of the side chain (Table I). One hydroxyl in the para position lowers Rf and shifts the ninhydrin-complex color to reddish brown. An additional hydroxyl group on the ring lower the Rf further as shown for the two methyl substituted compounds. The lowest Rf was obtained with norepinephrine which gives a brown color. Substitution of the ring hydroxyls with methoxy groups increases the Rf while substitution of the aliphatic hydroxyl with a carbonyl as in arterenone makes the ninhydrin-complex bright orange. A hydroxyl alpha to the amine group does not alter the usual purple color seen in the aliphatic series when there are no substitutions on the ring. Both histamine and tryptamine can be distinguished from the simple and phenyl-substituted aliphatic compounds by their brownish-red colors as well as by their Rf values.

Sensitivity of Primary Amino Groups to Ninhydrin.—Fitzpatrick's[37] use of pyridine with ninhydrin for amino acids was found to have an advantage for amines in the test tube as well as in chromatography. All compounds containing an $-NH_2$ group do not form colors with ninhydrin. However, no secondary or tertiary amines or other compounds have been discovered which will give the ninhydrin reaction under the conditions used. Proline, a possible exception, may develop its color through tautomerism with a split ring. Table III lists the types of compounds tested according to their structural formulas and whether or not they give the ninhydrin reaction. Examples falling in each group are also given. Primary amine groups attached directly to rings, amides, and secondary and tertiary amines with few exceptions give no color. A few amines differ from the others in requiring special treatment to obtain their optimum color development. In general these are of the $R-\underset{\displaystyle R'}{\underset{|}{C}}H-NH_2$ and the $R-\underset{\displaystyle O}{\underset{\|}{C}}-CH_2NH_2$ types, but methylamine also falls in this category.

TABLE III. REACTION OF NINHYDRIN WITH NITROGEN COMPOUNDS IN PRESENCE OF PYRIDINE

REACTION RATE	TYPE OF COMPOUNDS*	EXAMPLES
Rapid	$R-\underset{\displaystyle H}{\underset{|}{C}}-NH_2$	Ethylamine, butylamine, isoamylamine, hexylamine, heptylamine, decylamine, dodecylamine, tetradecylamine octadecylamine, tryramine, tryptamine, histamine, arterenol, glucosamine, glutamine, 2-phenylethylamine. Exception: Methylamine.
	$R-\underset{\displaystyle NH_2}{\underset{|}{\overset{\displaystyle H}{\overset{|}{C}}}}-COOH$	Glycine, alanine, leucine, methionine, phenylalanine, tryptophane, tyrosine, histidine, lysine, arginine, ornithine citrulline, glutamic acid, aspartic acid.
Retarded	$R-\underset{\displaystyle R'}{\underset{|}{\overset{\displaystyle H}{\overset{|}{C}}}}-NH_2$	1,3, dimethyl pentylamine; 1-methyl heptylamine; 1-methyl 2-(4-hydroxyphenyl) ethylamine; 1-butyl,2-hydroxy 2-phenylethylamine; 1-phenylethylamine, cyclohexylamine. Exception: Amino acids.
	$R-\underset{\displaystyle O}{\underset{\|}{C}}-\underset{\displaystyle H}{\underset{|}{C}}-NH_2$	α-dimethyl amino, 2,4-dihydroxy acetophenone; α-amino 3,4,-dihydroxy acetophenone.
	Others	Methylamine. Proline.
No reaction	$-NH_2$	Aniline, p-bromoaniline, p-nitroaniline, adenine.
	$R-\underset{\displaystyle O}{\underset{\|}{C}}-NH_2$	Urea, acetamide.
	$R-\underset{\displaystyle R'}{\underset{|}{N}}-H$	Epinephrine, xanthine. Exception: Proline.
	$R''NH_2$	Ammonia, hydroxylamine.

*R and R′ represent any organic substituents; R″ represents OH or H.

Quantitative Measurement.—Quantitation of known isolated amines, preparation of standard concentration curves, and absorption spectra of ninhydrin amine complexes in the visible range may be studied as follows: Prepare standard dilutions of amine to be studied to contain between 2 and 20×10^{-4} millimoles per millimeter. Dilute unknown with acid-isopropanol to be within this range. Pipette 1 ml. of each standard into a Pyrex tube of 30 ml. capacity, add 5 ml. of pyridine, 2 ml. of ninhydrin reagent, and acid-isopropanol mixture quantitatively to make a total of 10 milliliters. Use not more than 3 ml. of unknown and treat similarly. A blank is prepared by substituting the acid-isopropanol for amine

solution. Mix thoroughly, avoiding contact of solution with stoppers or skin, place in water bath at 85° C. for exactly seven minutes, remove rack from bath, and immerse immediately in cold water. Color intensity can be read over a period of one hour without appreciable fading.

Although all aliphatic amines found to react with ninhydrin have had major peaks in the 415 and 575 mμ ranges, distinct and consistent differences in both visual appearance and the shape of the absorption curves of the aromatic and heterocyclic amines occur. Fig. 2 shows the curves for a few amines of potential biological interest. In general, ring compounds and those containing -OH groups transmit more yellow and therefore appear brown

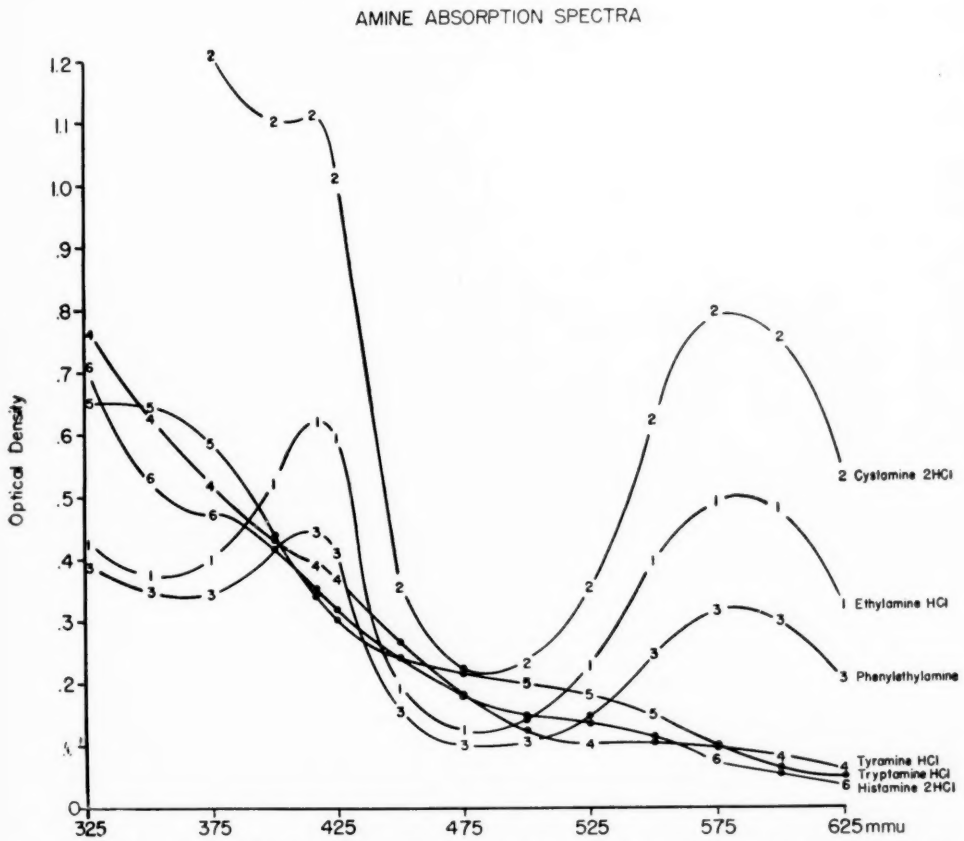

Fig. 2.—Spectral absorption curves for ninhydrin-amine complexes of selected primary amines as shown. Concentration of amines in the colored solutions in each case was 1.00 × 10^{-4}M. Experimental values are represented by numbers or dots according to space available. Double peak is characteristic of simple aliphatic compounds and α-phenylethylamine, and single peak of other aromatic and heterocyclic compounds.

to brownish-purple. Arterenone gives a definite orange color. Aliphatic amines are blue in dilute solution and become increasingly purple in higher concentrations. These differences clearly show up in the absorption spectra and are useful in the identification of amines on paper in chromatography discussed above.

Standard Concentration Curves.—At a wave length of 575 mμ and a slit width of 1.0 mm. on the Beckman spectrophotometer the spectral band width is 30 mμ. This is wide enough to include the peaks of all the amines studied and might be used as a rough index of the amine concentration of a mixture. As seen in Fig. 3, however, the range in extinction

coefficient of the concentration curves is large, so that an error of 50 per cent in such an estimation is possible. The relative extinction coefficients of the compounds studied will be discussed in greater detail in the second paper of this series.[38] The low slopes obtained for methylamine and butylamine may be partially due to the fact that they were not hydrochlorides. Volatilization may have occurred in the alkaline pyridine solution before the amines were bound in the ninhydrin complex.

Fig. 4 shows that similar results may be obtained with amino acids as with amines and that distinguishing characteristics also appear in this type of compound when pyridine is used.

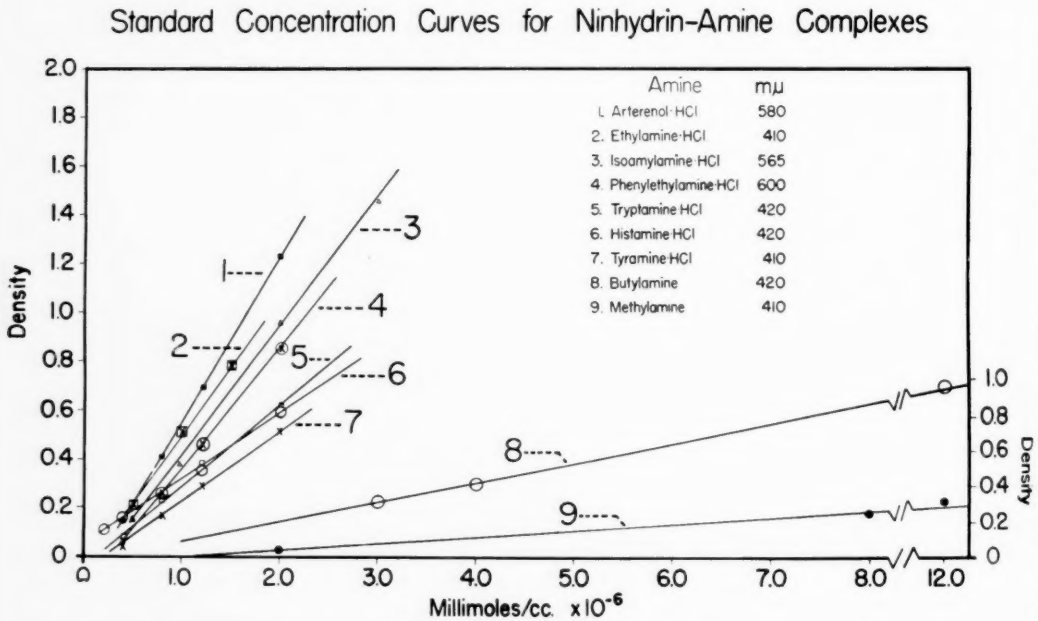
Standard Concentration Curves for Ninhydrin-Amine Complexes
Amine mμ
1. Arterenol·HCl 580
2. Ethylamine·HCl 410
3. Isoamylamine·HCl 565
4. Phenylethylamine·HCl 600
5. Tryptamine·HCl 420
6. Histamine·HCl 420
7. Tyramine·HCl 410
8. Butylamine 420
9. Methylamine 410
Density
Millimoles/cc. x 10⁻⁶
0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 12.0
0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6 1.8 2.0

Fig. 3.—Standard concentration curves for ninhydrin-amine complexes. These curves must be prepared for each set of determinations. Variations in purity of ninhydrin and pyridine, and water content of the stock solutions affect their slopes. See text.

DISCUSSION

Nonspecific methods have been used for determination of basic amines in blood. As measured by Richter's method,[25] the picrate color produced by certain organic bases is more intense in blood of hypertensive than of normotensive subjects.[1] Similarly, substances forming colored complexes with methyl orange presumably have a concentration higher than normal in patients with nephrosclerosis and other metabolic diseases.[28] The lack of specificity of these methods makes it impossible to know whether an alteration in the concentration of the same substance has occurred or whether one base has been replaced by another with a different molecular extinction coefficient. Since each of the above reagents reacts with a large number of unrelated compounds, the metabolic defects implicated by their reactions remain remote unless separation of the reacting metabolites can be achieved.

An extensive literature has grown up on the use of ninhydrin[39, 40] in the demonstration of amino acids on paper chromatograms and on their quantitative determination. The paucity of amines found in urine by these means is explained

by the fact that these methods have not been as sensitive for amines as for amino acids. The methods described in this paper for the quantitation of these substances will be useful in the study of metabolic patterns in hypertension and other disease states as well.

SUMMARY

Methods are described for isolation of primary amines on paper chromatograms, their partial identification, and methods of quantitative determination of known primary amines.

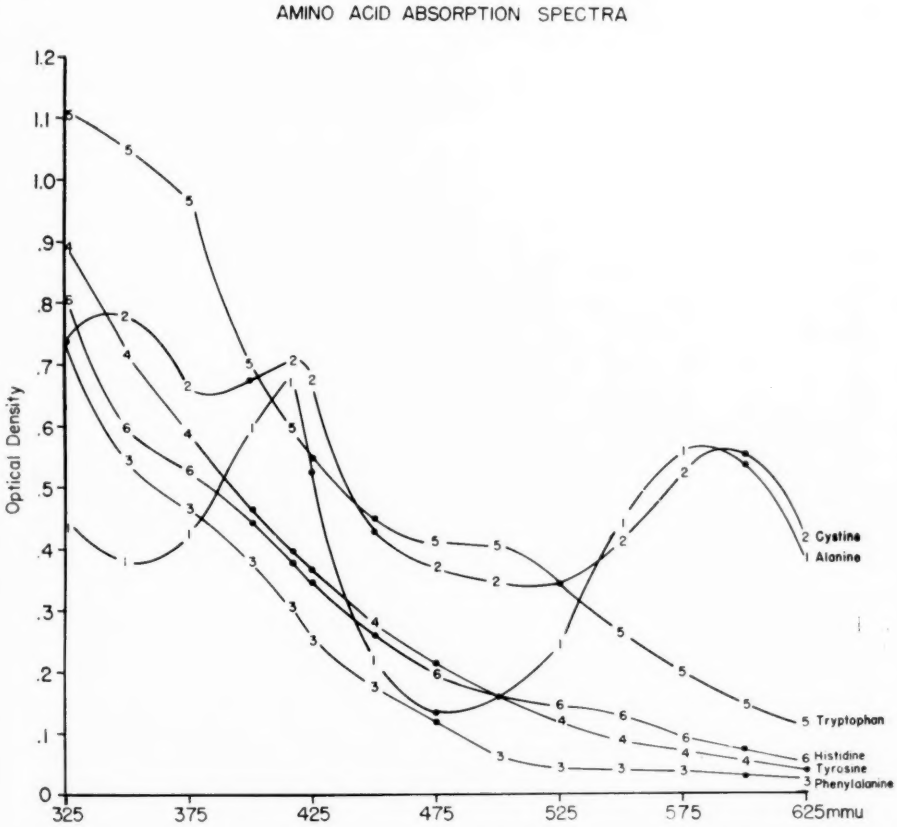

Fig. 4.—Spectral absorption curves of ninhydrin-amino acid complexes. Concentration of amino acids in the colored solutions in each case was 1.00×10^{-4}M. Experimental values are represented by numbers or dots according to space available. Note qualitative agreement of spectral curves of amino acids with their amine analogs.

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THE QUANTITATIVE DETERMINATION OF STREPTOKINASE

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THE assay for streptokinase, the activator of the proteolytic enzyme, plasmin, is an indirect assay, since its activity is measured in terms of the activity of the enzyme. The natural substrate for this enzyme is fibrin, but gelatin and casein[1-3] are also degraded, and a number of assay procedures have been proposed using all of these proteins as the substrate for plasmin activity.[2, 3] As a measure of the end point in the streptokinase assays, methods have been used which involved the physical degradation of the protein substrate[4-6] or the chemical determination of the various end products formed by the proteolytic activity of plasmin.[2, 7] The definition of the streptokinase unit has varied with the assay procedure used and no correlation among the various methods has been made. Christensen[8] proposed a streptokinase unit, based on the total clot lysis time, that has been adopted by several laboratories. However, certain difficulties appear in this method as well as in some of the other proposed assay procedures. Replicate assays performed on the same lot of streptokinase indicated that the unit obtained by the Christensen double-dilution assay depended on factors that are difficult to control, such as: (a) the plasminogen preparation, (b) age of the plasminogen solution, (c) ability of the assayist to read the end point, and (d) the duration of the assay. Since streptokinase is used clinically, a more precise determination of the dosage to be used in therapy was felt to be desirable.

The method described in this paper was developed to reduce to a minimum the recognized sources of technical error, and to place the assay on a standardized basis in conformity with the fundamental principles of biological assays.

MATERIALS AND METHODS

Streptokinase Standard.—A purified preparation of streptokinase was obtained from the culture medium of a *Streptococcus hemolyticus* H46A fermentation. After repeated assay by the Christensen method the preparation was assigned a value of 50 units per milligram and the lot was designated as standard streptokinase. It should be recognized that this assigned value is quite arbitrary and has no implications of establishing a general unit of streptokinase activity.

Plasminogen.—A lot of plasminogen was prepared in these laboratories by Dr. W. Baumgarten, using the Harvard method of fractionation of human plasma.[9] Preformed plasmin, which interferes with the assay, was present in only minor quantity. To prevent spontaneous activation of plasminogen while in solution, the required amount of plasminogen is weighed out prior to performing the assay, and is restored in sufficient borate buffer to make a 0.1 per cent solution just before using. The plasminogen stock is kept in the desiccator at 5° C.

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Fibrinogen.—Armour's Bovine Fraction I is used as the source of fibrinogen. This preparation is used without any pretreatment and is stored in the desiccator at 5° C. The required amount of fibrinogen is weighed out before beginning the assay and is restored in sufficient borate buffer to make a 0.25 per cent solution just before use.

Thrombin.—Parke, Davis & Company Bovine Thrombin is restored in 50 per cent borate buffer—50 per cent glycerol to contain 1,000 NIH units per milliliter. This stock solution can be stored at 5° C. A working solution of thrombin is prepared by making a 1:40 dilution of the stock solution in borate buffer. This dilution was found to be correct for several lots of thrombin purchased from Parke, Davis & Company; however, the concentrations may be altered, if necessary, so that 0.1 ml. of the working thrombin solution will clot the standard fibrinogen system in 15 to 30 seconds.

Borate Buffer.—The borate buffer consists of:

2.5 per cent H_3BO_3	45 parts
0.5 per cent NaCl	45 parts
4.0 per cent $Na_2B_4O_7$	10 parts

Gelatin Buffer.—Five-tenths per cent Eastman calfskin gelatin is dissolved in a saline buffer (0.9 per cent NaCl in 0.1M KH_2PO_4, pH 7.4). The solution is dispensed in 200 ml. quantities and sterilized at 121° C. for twenty minutes.

RESULTS

Reagents as a Source of Variation in the Streptokinase Assay.—During the early work on the streptokinase assay in this laboratory, a series of streptokinase samples were sent to several laboratories for collaborative assays. The divergence in the reported results was marked. Since essentially the same assay procedure was employed in the various laboratories, the differences in the values suggested that variation in the reagents used in the assay might be an important factor in the lack of correlation among the assays. In order to study this further, a complete set of reagents (Lot A) was obtained from another laboratory labeled as follows: *Bovine Fibrinogen*—Armour's Fraction I; *Plasminogen*—Harvard Fraction III (the streptokinase assay values to be multiplied by a factor of 2.6 to compensate for inhibitory substances in the plasminogen); *Thrombin*—clotting globulin. A streptokinase preparation was obtained through the courtesy of Dr. R. Murray of the National Institutes of Health labeled "Reference SK-SD —Streptokinase: 70,000 units; Streptodornase: 20,000 units." Christensen's double dilution method was used as the assay procedure. The NIH reference streptokinase was assayed with the two systems—one utilizing all the reagents from Lot A, the other prepared with the reagents normally used in this laboratory (Lot B). Assays were then performed with the two systems, interchanging the Lot A and Lot B reagents one at a time. The results are shown in Table I. With the complete Lot A system the contents of the vial of reference streptokinase is reported at 30,000 units—2.5 times less than the labeled contents. The vial assayed at 156,000 units with the Lot B system, or 2.2 times more than the labeled contents. Using Lot B plasminogen in the Lot A system increased the unitage of the streptokinase to approximately the labeled potency (72,000 units), but placing the Lot A plasminogen in the Lot B system has little effect on the assay (159,000 units, after correction by the 2.6 factor). When the fibrinogens were interchanged, the Lot B fibrinogen raised the assay values to 83,500 units,

while the other fibrinogen decreased the values as assayed by the Lot B system to 78,000 units. Both values were thus approximately equal to the labeled potency of the reference. Interchange of the thrombins had little effect on the values obtained by either system, both approximating the controls. Thus it appeared that the fibrinogen and the plasminogen preparations may be important factors in establishing reproducible and accurate assays.

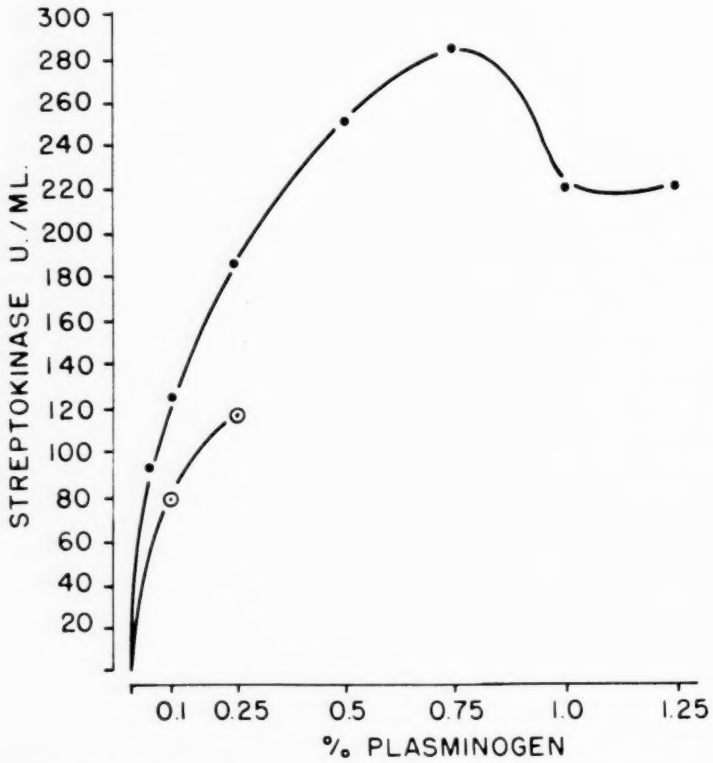

Fig. 1.—The effect of the plasminogen preparation and concentration on streptokinase assay values. ⊙———⊙ Plasminogen preparation obtained from an outside source; •———• Lot B plasminogen (see text).

Further evidence of the variability in plasminogen preparations is shown in Fig. 1. Plasminogen obtained from another source and the Lot B preparation were titrated against a constant amount of streptokinase. The results indicate that with the Lot B preparations the values of the streptokinase assay are dependent on the plasminogen concentration p to 0.75 per cent; beyond this concentration the assays become unreliable due to the excess of plasminogen present. Concentrations beyond 0.25 per cent of the other plasminogen could not be used because the particulate nature of the suspension interfered with the assay. The Lot B preparation also appeared to contain more available plasminogen, yielding higher streptokinase values than equal concentrations of the other preparation.

TABLE I. THE EFFECT OF THE REAGENTS ON THE STREPTOKINASE ASSAY VALUES

PLASMINOGEN	FIBRINOGEN	THROMBIN	STREPTOKINASE UNITS/VIAL	STREPTOKINASE UNITS CORRECTED BY 2.6 FACTOR
Lot A	Lot A	Lot A	14,000	30,000
Lot B	Lot B	Lot B	156,000	--
Lot A	Lot B	Lot A	32,000	83,500
Lot B	Lot A	Lot B	78,000	--
Lot A	Lot A	Lot B	18,900	49,000
Lot B	Lot B	Lot A	144,000	--
Lot B	Lot A	Lot A	72,000	--
Lot A	Lot B	Lot B	61,200	159,000

It appears, therefore, that in order to obtain more reproducible values of the Christensen unit among laboratories, or even within a laboratory, it is necessary to standardize all reagents used. This can be done quite easily by titrating the various reagents against a reference streptokinase until concentrations are reached which yield the expected streptokinase unitage. Experience with the method also points up very strongly the need for a reference streptokinase, and the adoption of a procedure in which the potency of an unknown preparation is determined in relation to a reference standard.

Assay Procedure: Preparation of Samples.—A quantity of standard streptokinase is diluted in gelatin buffer to yield a solution containing 100 units streptokinase per milliliter. A guess of the potency of the unknown (test) preparation is made and the sample is diluted in gelatin buffer to contain approximately 100 units per milliliter. Each preparation to be assayed is then diluted 1:5, 1:8.8, 1:15.3, and 1:26.8 in the following manner.

To a series of four tubes for each preparation to be assayed are added 2.0, 3.9, 4.3, and 7.7 ml. gelatin buffer, using the same 5 ml. pipette throughout the operation. With a one milliliter pipette, 0.5, 0.5, 0.3, and 0.3 ml. of the streptokinase preparations are added to the gelatin buffer dilution blanks in the order indicated above. The same pipette is used to dispense the streptokinase standard and the test preparations. Between preparations the pipette is rinsed several times with distilled water, alcohol rinsed, and hooked onto a vacuum line to dry. One-tenth milliliter aliquots of the streptokinase dilutions are transferred to the assay tubes (10×70 mm. Kahn serological test tubes), starting with the highest dilution in the series (1:26.8), and then in order of increasing concentration. A single 0.2 ml. pipette, washed and dried between preparations as above, is used for this procedure.

In order to save time in this initial step of the assay, it has been found advisable to prepare the streptokinase standard dilutions first, then, while the pipette is drying, to transfer the 0.1 ml. aliquots. This procedure of alternately diluting and transferring is continued for all test preparations.

The assay tubes at this point contain 0.1 ml. aliquots of each of four dilutions for the streptokinase standard and each test preparation. To every tube is now added, from a 2.0 ml. pipette, 0.4 ml. of the freshly restored fibrinogen, so the corresponding tube in each series receives the fibrinogen from the same portion of the pipette. The plasminogen is restored and 0.5 ml. quantities are

added to each tube from a 2.0 ml. chemical pipette at 5 second intervals on a stopwatch. Immediately thereafter, also at 5 second intervals, 0.1 ml. thrombin is added to each tube from a 2.0 ml. pipette graduated in 0.01 ml. Upon the addition of thrombin to each tube, it is shaken thoroughly and placed in a rack in the water bath at 35° C. Thirty seconds after the thrombin is added to the last tube, the tube is tilted gently to confirm the formation of the clot. The stopwatch is reset and the tubes are observed for lysis of the clots. Clot lysis is determined by the rise of the air bubbles formed as the clot softens, and the end point is taken as the time (to the closest fifteen seconds) the last moving bubble enters the meniscus. Tubes should not be disturbed in the water bath as it has been found that agitation tends to induce the formation of fibrin fibers which interfere with the movement of the bubbles.

TABLE II. TRANSFORMED LYSIS TIMES

MINUTES	SECONDS			
	0	15	30	45
5	69.9	72.0	74.0	76.0
6	77.8	79.6	81.3	82.9
7	84.5	86.0	87.5	88.9
8	90.3	91.7	92.9	94.2
9	95.4	96.6	97.8	98.9
10	100.0	101.1	102.1	103.1
11	104.1	105.1	106.1	107.0
12	107.9	108.8	109.7	110.6
13	111.4	112.2	113.0	113.8
14	114.6	115.4	116.1	116.9
15	117.6	118.3	119.0	119.7
16	120.4	121.1	121.7	122.4
17	123.0	123.7	124.3	124.9
18	125.5	126.1	126.7	127.3
19	127.9	128.4	129.0	129.6
20	130.1	130.6	131.2	131.7

INITIAL DILUTION OF TEST PREPARATION (D) = 2

DILUTION	STANDARD		TEST PREPARATION	
	LYSIS TIME (MIN.)	TRANSFORMED LYSIS TIME	LYSIS TIME (MIN.)	TRANSFORMED LYSIS TIME
1:26.8	$S_1 = 15\frac{1}{2}$	119.0	$U_1 = 15$	117.6
1:15.3	$S_2 = 12\frac{3}{4}$	110.6	$U_2 = 10\frac{1}{2}$	102.1
1:8.8	$S_3 = 8\frac{3}{4}$	94.2	$U_3 = 8$	90.3
1:5.0	$S_4 = 6\frac{3}{4}$	82.9	$U_4 = 6\frac{1}{2}$	81.3

Example: A Single Test Preparation Assay.—*

$$V = U_4 + U_3 + U_2 + U_1 - S_4 - S_3 - S_2 - S_1 = -15.4$$
$$B = 3U_4 + U_3 - U_2 - 3U_1 + 3S_4 + S_3 - S_2 - 3S_1 = -245.4$$
$$P = 3U_4 + U_3 - U_2 - 3U_1 - 3S_4 - S_3 + S_2 + 3S_1 = 4.0$$
$$T = U_4 + U_3 + U_2 + U_1 + S_4 + S_3 + S_2 + S_1 = 798.0$$
$$T_i^2 = U_1^2 + U_2^2 + \text{----------} + S_3^2 + S_4^2 = 81{,}157.36$$
$$S^2 = (T_i^2 - T^2/8 - V^2/8 - B^2/40 - P^2/40)\ /4 = 21.8\ /4 = 5.32$$

*Calculations have been determined for an assay in which two test preparations are assayed simultaneously against the standard streptokinase. These computations are more cumbersome. For clarity, only the procedure for the single test preparation assay is presented in this paper. However, the authors will furnish the details of the extended assay upon request. Calculations can be made to cover any number of test preparations used in the assay, following recognized statistical procedures.[10]

Since the thrombin was added to each tube at five-second intervals, it is necessary to correct the lysis time of each tube by the addition of the appropriate number of seconds.

The data thus obtained, if plotted on log-log paper, would show a linear dilution-response curve for the standard streptokinase preparation and for the test preparation. To estimate the relative potency of the test preparation in terms of the standard it is necessary to determine the displacement between the two curves. To make this estimation reliable it must be demonstrated statistically that the two curves are parallel.

The statistical analysis for the validity of the assay and the determination of the relative potency of the test preparation requires the conversion of the observed lysis times (corrected for the difference in starting times) to their logarithms. In order to facilitate the calculations, these transformed responses are then multiplied by the factor 100. In Table II are listed the corrected transformed responses for the lysis times within the range five to twenty minutes.

These time limits have been selected because repeated assays have shown that the regression of log lysis time upon log dilution is linear in the time range five to twenty minutes. Assays in which one or more tubes lyse outside these time limits generally show curvature and apparent lack of parallelism between the standard and test preparation. Such assays are repeated after appropriate adjustment of the initial guessed dilution.

The test for parallelism between the standard and test preparation is made by computing $F = (P^2/40)/s^2 = (16/40)/5.32 = 0.08$. A value of F equal to or greater than 7.71 indicates nonparallelism ($p = 0.05$; odds of 1:20), and the assay should be repeated.

Since in this example there is no indication of a lack of parallelism, the relative potency and the approximate 95 per cent confidence limits of the assay may be determined. The pooled estimate of residual error (based on 5 degrees of freedom) is found to be $s_0^2 = (4s^2 + P^2/40)/5 = 21.68/5 = 4.34$.

Relative Potency (R. P.) $= D \text{ antilog } (2 + 1.215\ V/B)$
$= (2) \text{ antilog } [2 + (1.215)(-15.4)/(-245.4)]$
$= 2 \text{ antilog } 2.0762 = 2\ (119)$
$= 238$ per cent of standard

95 per cent Confidence Limits

$$= R.P. \pm \frac{20.3\ (R.P.)\ s_0 \sqrt{1 + 5V^2/B^2}}{B}$$

$$= 238 \pm \frac{(20.3)\ (238)\sqrt{4.34}\ \sqrt{1 + 5(15.4)^2/(245.4)^2}}{245.4}$$

$= 238\% \pm 44\% = 194\%$ to 282% of Standard

In a previously designed assay method duplicate tubes were run at high and low concentrations of streptokinase but the intrinsic error calculated from the difference between duplicate tubes did not reflect the true error of the assay. There was enough variation among, and even within, the nonvolumetric pipettes used for dilutions to interject a bias in the results. Estimates of potency and tests for the validity of the assays consequently were biased also. Differences in

exposure time and temperature of the reaction constituted an additional source of variation. Taking all these factors into consideration, the multi-level, single tube assay described above was devised wherein an attempt was made to control all these recognizable sources of error. The residual error of this assay is derived from the deviation of the experimental results from those postulated by the linear regression of log lysis time upon log dilution. Confidence limits estimated from this source of variation are wider and more realistic than those derived from the discrepancy between duplicate tubes, and will include more estimates of relative potencies of repeat assays, which often was not the case in the duplicate tube method.

This assay has been found to be a satisfactory method for the screening of crude streptokinase preparations, some of which may contain modifying substances inducing a potentiation or inhibition of the enzyme or its activator. It is often possible to detect the presence of these substances by the difference in the slope of the response curves of the standard and unknown preparations. The assay will appear invalid, and should confirmation be obtained on repeating the assay, the preparation should then be subjected to further purification.

SUMMARY

The assay of streptokinase by a relative potency method is described and suggested for use. This method, when used with standardized reagents and a reference streptokinase preparation, eliminates many sources of error inherent in previous procedures. The statistical treatment of the data permits an estimate of potency to be obtained together with 95 per cent confidence limits of the error of the assay and provides means for detecting significant deviations from parallelism. The latter is useful for detecting certain interfering substances that may occur in streptokinase preparations.

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THE DETERMINATION OF SERUM ALKALINE PHOSPHATASE ACTIVITY

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INTRODUCTION

A NEW and simple azo dye method for the determination of phenol in biologic material was introduced by Gomori[1] in 1949, who then made this the basis for the measurement of certain enzyme activities. Various phenyl esters were employed as substrates and the amount of free phenol liberated by enzymatic hydrolysis was determined. The activities of serum alkaline and acid phosphatase were assayed in this manner, using disodium phenylphosphate as a substrate. In addition to the sensitive, simple method for the determination of phenol, another advantage of Gomori's method over some widely used procedures is the fact that serum proteins do not have to be precipitated.

In order to adapt Gomori's method[1] for the routine determination of serum alkaline phosphatase, it was necessary to introduce several modifications. The principal change consists of employing a chemical inhibitor, formaldehyde, to stop the enzymatic hydrolysis at the end of the incubation period instead of immersing the reaction tubes in ice water. The reaction is stopped instantly and completely by the inactivator for at least six hours. The use of ice in a routine laboratory is unsatisfactory, since the inhibition of the reaction is not instantaneous, and the development of color must then proceed without interruption. Some changes have been made in the volumes of reagents involved in the assay so that all operations, namely, incubation, color development, and color reading can be carried out in a single test tube. The time of incubation was decreased from sixty minutes to thirty minutes, since the latter period is sufficiently long to produce measureable hydrolysis, there is less danger of inactivating the enzyme in the alkaline medium, and results can be obtained more quickly and conveniently.

As described below, 0.5 ml. of serum, which is diluted to 50 ml. before using, is normally required for the measurement of the activity of alkaline phosphatase. A slightly modified method is also presented in which the serum requirement is only 0.010 milliliters. The pipette is the only piece of ultramicro equipment required for this assay, since the remainder of the procedure is the same as when using larger amounts of diluted serum.

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MATERIAL AND METHODS

The reagents are the same strength as described by Gomori[1] with the exception of the diazo reagent. The formaldehyde solution is an addition.

1. *Substrate:* 0.05M disodium phenylphosphate.* Purify by extracting 1.5 Gm. with two 25 ml. portions of hot alcohol. Filter with suction and dry in air. Place 1.09 Gm. in a 100 ml. volumetric flask and make up to 100 ml. volume with water. It will keep for a year when stored in a refrigerator.
2. *Borate Buffer, pH 9.8:* Dissolve 9.5 Gm. of $Na_2B_4O_7 \cdot 10$ HO in 950 ml. of water and add 35 to 40 ml. of normal NaOH until a pH of 9.8 is reached, as registered on a pH meter. Make up to 1,000 ml. volume.

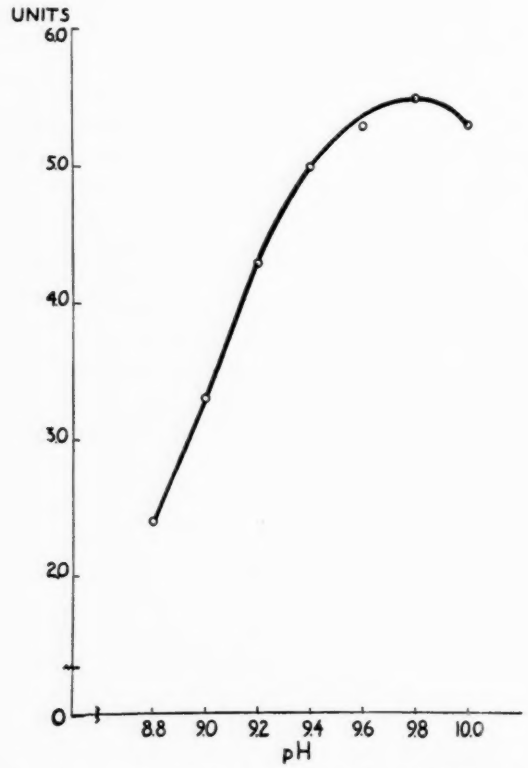

Fig. 1.—The effect of pH upon the activity of serum alkaline phosphatase.

3. *20 Per Cent $MgCl_2$:* Twenty grams of $MgCl_2$ are dissolved in water and made up to 100 ml. volume.
4. *Buffered Substrate:* To 10 ml. of substrate solution, add 1 ml. of 20 per cent $MgCl_2$ and make up to 100 ml. volume with borate buffer. The solution is stable for several months if stored in a refrigerator.
5. *1.5M Formaldehyde:* Dilute 120 ml. of 38 per cent formaldehyde to 1,000 ml. volume with water.
6. *Saturated Alcoholic Borax Solution:* To 150 ml. of 95 per cent ethyl alcohol, add 850 ml. of water and 35 Gm. of $Na_2B_4O_7 \cdot 10$ H_2O and shake well. Let the undissolved residue remain in the bottle.

*Eastman Kodak Co., Rochester, N. Y., or the Matheson Co., East Rutherford, N. J.

7. *Diazo Reagent:* Place 100 ml. of water in an ice bath and cool below 5° C. Dissolve 0.125 Gm. of Red B Salt* in the cold water and filter. Add 1 ml. of cold, approximately 1.8N H_2SO_4 (dilute 5 ml. concentrated acid to 100 ml.) and store in refrigerator. It will keep for two weeks in the cold.
8. *Standard Phenol Solution:* Dissolve 0.940 Gm. of anhydrous phenol crystals in water and make up to 100 ml. volume. Phenol concentration is 100 micromols per milliliter.
9. *Working Phenol Standard:* 0.05 micromols per milliliter. Dilute 1 ml. of standard phenol solution to 100 ml. volume. Transfer 5 ml. of this diluted standard to a 100 ml. volumetric flask and make up to volume with water. Prepare the working standard on the same day as used.

Macro Method.—A 1:100 dilution of serum is employed. Dilute 0.5 ml. of serum to 50 ml. with water. Smaller quantities, such as 0.1 ml. serum diluted to 10 ml., may be used if desired.

Place 2 ml. of buffered substrate in a test tube and allow to come to equilibrium in a water bath at 37° C. Add 1 ml. of diluted serum, mix, and let stand in 37° C. water bath for thirty minutes. At the end of this time, stop the reaction by adding 2 ml. of 1.5M formaldehyde solution and remove from the water bath.

A blank is run for each serum. Two milliliters of substrate are heated for thirty minutes at 37° C. and then 2 ml. of formaldehyde solution, followed by 1 ml. of diluted serum, are added.

The color in both test solution and blank is developed by the addition of 4 ml. of saturated alcoholic borax solution, followed by 1 ml. of diazo reagent to each. The solution must be mixed well immediately after the addition of the diazo reagent or otherwise the coupling of phenol with diazotized salt will be incomplete. After five minutes, the color of the test solution is read against its respective blank in a photoelectric colorimeter, using a filter that gives maximum transmission at 490 mμ. The color is stable for at least sixty minutes.

Alkaline phosphatase activity is obtained directly in units from a standard curve. The unit is defined by Gomori[1] as that activity which liberates one micromol of phenol per milliliter of serum per hour at 37° C.

Preparation of Standard Curve.—Place 0.5, 1.0, 2.0, 3.0, and 4.0 ml. of phenol working standard containing 0.025, 0.050, 0.100, 0.150, and 0.200 micromols, respectively, of phenol in a series of test tubes. Add 1 ml. of borate buffer and sufficient water to each to make a total volume of 5 milliliters. Develop and measure the color of the phenyl dye as described above by the addition of alcoholic borax solution and diazo reagent.

Plot on semilogarithm paper the per cent transmittance against 200 times the concentration of phenol expressed as micromols. Thus, the serum alkaline phosphatase activity is obtained directly in units when the time of reaction is 0.5 hour and 1 ml. of a 1:100 dilution of serum (or 0.01 ml. of serum taken directly) is employed.

Ultramico Method.—Serum alkaline phosphatase activity can be carried out directly with 0.01 ml. of serum with only a slight modification of the method. This is done as follows:

Place 2 ml. of buffered substrate plus 1 ml. of water in a test tube and bring to 37° C. Transfer 0.01 ml. of serum to this tube by means of an ultramicro pipette,† mix and incubate for thirty minutes at 37° C. The reaction is stopped by the addition of formaldehyde solution and color is developed as described above. A blank is prepared in a similar fashion by adding the serum after the formaldehyde solution. The same standard curve as developed for the macro procedure is employed.

*National Aniline Division, Allied Chemical and Dye Corp., New York, N. Y.
†Microchemical Specialties Co., Berkeley, Calif.

RESULTS AND DISCUSSION

Many compounds or ions have been reported as being able to inhibit the activity of alkaline phosphatase.[2, 3] Among these are cyanide, arsenate, phosphate, oxalate, bile acids, formaldehyde, ketene, phenyl isocyanate, amino acids, sulfhydryl compounds, and others. Gould[4] demonstrated that substances that block the free amino group of the enzyme are powerful inhibitors and that formaldehyde solution is quite effective. Some of these inactivators are not suitable for use in the assay of serum alkaline phophatase activity by the method described because they either fail to stop the enzyme reaction completely or produce interfering colors with the diazotized salt. A solution of 1.5M formaldehyde was selected as the one to stop the enzyme reaction because the effect is immediate and of long duration. In the presence of formaldehyde, serum phosphatase fails to hydrolyze measureable amounts of substrate even though incubated with the latter for several hours at 37° C. or six hours at room temperature. Formaldehyde does not interfere with the subsequent development of color produced by the formation of the phenyl dye.

It should be pointed out that the pH at which the assay is carried out, pH 9.8, is much higher than the ones employed by other methods. Gomori[1] reported that the pH optimum under the conditions of his method was pH 9.7 to 9.8 This has been confirmed in this laboratory, and a typical curve appears in Fig. 1. The pH optimum is approximately 9.8, and varies very little between pH 9.6 and 10.0.

The alkaline phosphatase activity of a number of human and dog sera was measured by both macro and ultramicro methods, and the results are reported in Table I. The assays were carried out in duplicate, and close agreement was obtained between the two methods. The per cent mean deviation between the two methods varied from a maximum of 4.1 per cent to a minimum of 0.0 per cent. The average per cent mean deviation was 1.4 per cent.

Since the unit of serum alkaline phosphatase activity as defined by Gomori[1] is different from those in common use and measured under different conditions, it is necessary to have well-established limits of normal for the method to be of clinical value. Gomori[1] reported a normal range of 2.3 to 6.6 units in 51 unselected human sera. He also found that 1 unit by his method is roughly equivalent to 1.2 Bodansky units.

In order to establish the normal range of serum phosphatase activity by the proposed method, the sera of two groups of individuals were obtained and analyzed. The first group consisted of hospital workers of both sexes who were in good health. As shown in Table II, the mean value for serum alkaline phosphatase activity in this group was 2.3 ± 0.9 units. The maximum and minimum values were 4.5 and 0.8 units, respectively. In contrast to this, the second group, consisting of 70 hospitalized patients without gross evidence of liver or bone disease or cancer, gave a mean value of 2.8 ± 1.03 units of serum alkaline phosphatase activity, with maximum and minimum values of 5.3 and 0.8 units, respectively. Since this group of hospitalized individuals had a higher level of enzyme activity, the upper limit of this group of subjects should be taken into

TABLE I. COMPARISON OF SERUM ALKALINE PHOSPHATASE ACTIVITY BY MACRO AND ULTRAMICRO METHODS

SERUM	SERUM PHOSPHATASE ACTIVITY*		PER CENT MEAN DEVIATION
	MACRO METHOD	ULTRAMICRO METHOD	
Human A	2.6	2.7	1.9
Human B	2.9	2.9	0.0
Human D	4.2	4.4	2.3
Human C	2.9	2.9	0.0
Human E	2.4	2.4	0.0
Human F	3.5	3.8	4.1
Human pooled No. 1	14.9	14.9	0.0
Human pooled No. 2	4.9	4.8	1.0
Dog. No. 1†	19.6	18.8	2.1
Dog. No. 2†	3.8	4.0	2.6
Dog. No. 3†	63.0	61.0	1.6

*The average of closely agreeing duplicate determinations.
†These dogs had ligated pancreatic ducts.

consideration when attempting to utilize the measurement of serum alkaline phosphatase activity in differential diagnosis. It is suggested that the normal range of activity be placed between 0.8 and 4.5 micromole units.

Serum alkaline phosphatase activity is elevated in two types of pathologic conditions: in bone diseases where there is increased osteoblastic activity and in various diseases of the liver and biliary passages. Values obtained by the new method parallel closely those reported for the Bodansky method.[5] In a limited time period in this hospital, the highest levels of serum alkaline phosphatase were found as follows: 166 micromol units in severe rickets, 61 units in Paget's disease and values of 57, 70, and 84 units, respectively, in three cases of carcinoma with metastases to bone.

When interpreted in conjunction with clinical symptoms and the results of other liver function tests, the level of serum alkaline phosphatase activity is of limited value in the differential diagnosis of liver disease. Where values of 10 or 15 Bodansky units are accepted as the arbitrary dividing line for diagnostic purposes,[6-8] this would become 8 and 12 micromol units, respectively, by the new method.

In the course of this investigation, an observation of Bodansky[9] that has not received sufficient attention in clinical laboratories was confirmed. The storage of serum in a refrigerator or deep freeze overnight resulted in an increase of serum phosphatase activity ranging from 5 to 30 per cent. Where sera are stored prior to assay, this increase in activity may be of significance in the interpretation of borderline cases.

TABLE II. SERUM PHOSPHATASE ACTIVITY OF HEALTHY AND HOSPITALIZED ADULTS

GROUP	NUMBER	SERUM ALKALINE PHOSPHATASE ACTIVITY (UNITS)		
		MEAN	MINIMUM	MAXIMUM
Normal, nonhospitalized	49	2.3 ± 0.89*	0.8	4.5
Hospitalized, without gross evidence of liver or bone disease or cancer	70	2.8 ± 1.03	0.8	5.3

*Standard deviation.

SUMMARY

A simple, reproducible method for the determination of serum alkaline phosphatase activity has been described. The measurement of enzyme activity may be carried out with as little as 0.01 ml. of serum. Normal values of serum alkaline phosphatase activity varied from 0.8 to 4.5 micromol units, with an average value of 2.3 ± 0.9 units.

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THE DETERMINATION OF SERUM ACID PHOSPHATASE ACTIVITY

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INTRODUCTION

IN THE preceding paper,[1] a modification of Gomori's method[2] for the determination of serum alkaline phosphatase activity was described. In a similar manner, this communication presents a modification of Gomori's method[2] for the estimation of serum acid phosphatase activity. The advantages of Gomori's techniques—the use of his sensitive method for the determination of phenol and the elimination of a step requiring protein precipitation—have been retained. The principal alteration consists of stopping enzymatic hydrolysis by means of a chemical inhibitor, sodium fluoride; changes have also been made in the volumes of reagents so that the assay and color development can be carried out in a single test tube.

Both macro and micro methods for the determination are described. The former requires 0.5 ml. of serum, whereas only 0.05 ml. are needed for the latter.

MATERIALS AND METHODS

The concentrated substrate solution, alcoholic borax solution, diazo reagent, and standard solutions are the same as those described for the determination of serum alkaline phosphatase activity.[1] In addition to these, the following reagents are necessary:

(a) *Acetate Buffer, 0.05M, pH 5.0:* Dissolve 4.75 Gm. of sodium acetate in about 950 ml. of water. Add approximately 0.9 ml. of glacial acetic acid and measure the pH. Add sufficient acetic acid to adjust the pH to 5.0 and then make the solution up to 1,000 ml. with water. Add several drops of toluene as a preservative, and store in a refrigerator.

(b) *Buffered Substrate:* To 10 ml. of substrate solution described previously,[1] add 90 ml. of acetate buffer. Store in refrigerator and discard after two or three weeks.*

(c) *Saturated Sodium Fluoride:* Stir 60 Gm. of sodium fluoride in 1,000 ml. of water and filter.

The procedure for measuring the activity of serum acid phosphatase differs from that of alkaline phosphatase in the following respects: (a) the serum dilution is 1:20 instead of 1:100, (b) the time of reaction is sixty minutes instead of thirty, (c) the pH employed is 5.0 instead of 9.8, (d) activation with magnesium chloride is not necessary, and (e) enzymatic hydrolysis is stopped by the addition of sodium fluoride instead of formaldehyde. The color of the liberated phenol is developed as described for alkaline phosphatase.[1]

Macro Method.—0.5 ml. of serum is diluted to 10 ml. volume with water. Two milliliters of buffered substrate are placed in a test tube in a water bath at 37° C. One milliliter of diluted serum is added and the mixture is kept at 37° C. for sixty minutes. The reaction is stopped by the addition of 2 ml. of sodium fluoride solution and the tubes are removed from the bath. A blank is prepared for each serum by incubating 2 ml. of substrate at 37° C. for sixty minutes and then adding the sodium fluoride, followed by 1 ml.

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*A mold containing an acid phosphatase may grow in this solution if precautions are not taken. Discard when blanks become high.

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of diluted serum. The azo dye of phenol is produced by the addition of alcoholic borax solution and diazo reagent. The intensity of the dye is obtained by measuring the optical density at 490 mμ in a photoelectric colorimeter.

A standard curve for acid phosphatase is prepared by utilizing the values previously obtained with the Phenol Working Standard.[1] The per cent transmittance is plotted on semilogarithm paper against twenty times the concentration of phenol expressed as micromoles. Thus, the activity of serum acid phosphatase is obtained directly in micromole units when the time of reaction is 1.0 hour and 1 ml. of a 1:20 dilution of serum (or 0.05 ml. of serum taken directly) is employed. One unit of phosphatase activity is that which liberates one micromole of phenol from the substrate per milliliter of serum per hour at 37° C.[2]

Micro Method.—Serum acid phosphatase measurement can be carried out directly, using 0.050 ml. of serum. The only modification in the procedure is to add 1 ml. of water to the 2 ml. of substrate and then introduce 0.05 ml. of serum by means of a self-adjusting micropipette* instead of 1 ml. of a 1:20 serum dilution.

RESULTS AND DISCUSSION

Several workers have demonstrated that the activity of acid phosphatase is inhibited by phosphate, arsenate, molybdate, fluoride, and oxalate ions.[3, 4] Sodium fluoride was shown to be strongly inhibitory for this enzyme prepared from various animal tissues by Monemura[5] and by Belfanti and associates,[6] while Gutman and Gutman[7] demonstrated the same type of inhibition for the acid phosphatase in serum. In the present study, the addition of a concentrated solution of sodium fluoride was adopted as the means of stopping the action of serum acid phosphatase because its effect is immediate and complete. In the concentration employed, sodium fluoride completely prevents enzymic hydrolysis of disodium phenylphosphate for at least three hours at room temperature and does not interfere with subsequent color formation.

The acid phosphatase activity of a number of human sera taken at random was measured by both macro and micro methods, and the results reported in Table I. The assays were carried out in duplicate and close agreement was obtained between the two methods. In 10 separate assays, the difference between the two methods varied from 0.00 to 0.03 unit. The standard deviation of the differences was 0.017 unit.

TABLE I. COMPARISON OF SERUM ACID PHOSPHATASE ACTIVITY BY MACRO AND MICRO METHODS

HUMAN SERUM	SERUM PHOSPHATASE ACTIVITY		DIFFERENCE BETWEEN THE 2 METHODS (UNITS*)
	MACRO METHOD (UNITS*)	MICRO METHOD (UNITS*)	
1	0.59	0.59	0.00
2	0.52	0.54	0.02
3	0.57	0.58	0.01
4	0.55	0.54	0.01
5	1.08	1.08	0.00
6	0.38	0.41	0.03
7	0.46	0.46	0.00
8	0.67	0.65	0.02
9	0.21	0.24	0.03
10	0.37	0.37	0.00
Standard deviation of the differences			0.017

*Micromole units.

*Microchemical Specialties Co., Berkeley, Calif.

In order to establish the normal variation of serum acid phosphatase activity, the sera of 390 hospitalized adult, male patients who showed no signs of carcinoma of any type were assayed, and the results appear in Table II. The mean value for this group was 0.55 ± 0.32 micromole unit, with maximum and minimum values of 2.8 and 0.15, respectively. Only 4 patients of the 390, however, had enzyme levels exceeding 1.5 micromole units. The activities found in this laboratory are lower than the range of 0.64 to 1.48 units obtained by Gomori[2] on 51 unselected human sera. The reason for this difference is not known. Falsely high values may be obtained if the blood is hemolyzed, since erythrocytes contain an acid phosphatase,[5] while low values may be the result of partial inactivation of the enzyme if the serum should remain too long at room temperature before being analyzed. Taking the experience of both laboratories into consideration, it is proposed that 1.5 micromole units be established as the upper limit of normal and that values above this limit be considered pathologic.

Measurement of serum acid phosphatase activity is useful in the diagnosis of metastasizing cancer of the prostate and in following the effects of therapy. Sullivan, Gutman, and Gutman[7] have reviewed the theoretical and experimental developments that led to the use of the serum acid phosphatase determination in this disease. These workers noted that 85 per cent of patients with metastasizing prostatic cancer had serum acid phosphatase values above 3 King-Armstrong units, while 90 per cent of patients without prostatic disease had levels below 3 units. In our experience with the new method, 5 of 6 patients with prostatic carcinoma and roentgenologic evidence of metastases had acid phosphatase levels above 1.5 micromole units, as shown in Table II. Thus, with the limit of normal taken as 1.5 micromole units, one false negative for metastasizing carcinoma of the prostate was obtained in a small series of 6 patients with this disease, and 4 false positives were found in a series of 390 patients without cancer.

SUMMARY

A simple, reproducible method for the determination of serum acid phosphatase activity has been described. The measurement of enzyme activity may be carried out with as little as 0.05 ml. of serum. It is tentatively proposed that serum acid phosphatase levels above 1.50 micromole units be considered pathologic.

TABLE II. COMPARISON OF SERUM ACID PHOSPHATASE ACTIVITIES OF A GROUP OF PATIENTS HAVING NO EVIDENCE OF CANCER WITH A GROUP OF PATIENTS HAVING PROSTATIC CARCINOMA WITH METASTASES

GROUP	NUMBER IN GROUP	SERUM ACID PHOSPHATASE ACTIVITY			NUMBER WITH ACTIVITY ABOVE 1.5 UNITS	REMARKS
		MEAN (UNITS*)	MAXIMUM (UNITS*)	MINIMUM (UNITS*)		
Hospitalized adult males without cancer	390	0.55 ± 0.32†	2.8	0.15	4	1.0% false positives
Adults with metastasizing prostatic cancer	6	2.80	6.2	0.87	5	17% false negatives

*Micromole units.
†Standard deviation.

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